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Genetic Contributions to Susceptibility and Resistance to Learned Helplessness

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Craig Scott Garafola

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Abstract of the Dissertation

Genetic Contributions to Susceptibility and Resistance to Learned Helplessness

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Depression is a disease with a complex etiology that is only beginning to be studied from a genetic perspective. A selectively bred line of rats, susceptible to the learned helplessness model of depression, presents an opportunity to search for genes affecting either the depressive symptoms or the predisposition towards developing the disease. A microarray revealed a small set of genes with altered expression in the hippocampus of the congenitally helpless rats. We selected one of these genes, a member of the γ-protocadherin family, for further study to determine the basis for the change in expression. Helpless animals demonstrated an increased expression of protocadherin gamma A11, primarily in CA1 neurons. In addition, the helpless resistant line displayed changes in expression that favored a more excitotoxic state. Protocadherins have been implicated in synapse development and may alter the connections within the hippocampus in a way that enhances the correlates of depression found in other brain regions of the learned helpless rat. A greater understanding of the function of the clustered protocadherins in the establishment of neuronal networks is required to determine how these molecules affect the functional characteristics of the helpless brain.

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List of Abbreviations

LH, learned helpless;

NLH, non-learned helpless(LH resistant);

cLH, congenitally helpless;

cNLH, congenitally non-learned helpless;

GWAS, genome-wide association study;

GFAP, glial fibrilary acidic protein;

MAP-2, microtubule associated protein 2;

Pcdhga11, protocadherin gamma A11;

Kmo, kynurenine 3-hydroxylase;

Gsbs, G-substrate;

Slc35d3, solute carrier family 35 member D3;

LTP, long term potentiation;

LTD, long term depression;

cGMP, cyclic guanadine monophosphate;

qPCR, quantitative polymerase chain reaction;

NAD, nicotinamide adenine dinucleotide;

NMDA, N-methyl-D-aspartate;

NO, nitric oxide;

PKG, cGMP-dependent protein kinase;

DRN, dorsal raphe nuclei;

CpG, cytosineguanidine dinucleotide;

EC, entorhinal cortex;

CTD, C-terminal domain;

VCD, variable cytoplasmic domain;

PSD, post-synaptic density;

RNAi, RNA interference;

PSD-95, postsynaptic density protein of 95kD;

CaMKII-α, calcium-calmodulin dependent kinase 2 alpha subunit;

CaMKII-γ, calcium-calmodulin dependent kinase 2 gamma subunit;

SNIP, SNAP-25 interacting protein;

pcdh, protocadherin;

LAS AF, Leica Application Suite Advanced Fluorescence;

FBS, fetal bovine serum;

NDS, normal donkey serum;

RMA, Robust Multi-array Averaging;

CA1, cornu ammonis area1;

CA3, cornu ammonis area 3;

BNST, bed nucleus of the stria terminalis;

LHb, lateral habenula

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Chapter 1: Background and Significance

Introduction

The World Health Organization Global Burden of Disease Study ranked major depressive disorder as 11th greatest cause of ill health in the world, representing a 37% increase in disease burden created by this disorder (Murray et al. 2012). In high income North America, depression jumps to the 5th rank. As a condition, it is defined by the DSM-IV as having (1) a depressed mood or loss of interest or pleasure in normally pleasurable pursuits and (2) four more symptoms of depression, which include the above two symptoms, anxiety or guilt, suicidal thoughts or attempts, sleep disturbances, impaired concentration, fatigue, psychomotor retardation or agitation, and changes in appetite or weight. These must be persistent and have a significant effect on the patient's ability to function. While the acute effects of treatments are known, the mechanisms of by which they mitigate the symptoms of depression is still an area of active study. Furthermore, the etiology of depression, while recognized as diverse, is not well understood, despite decades of research (Krishnan & Nestler 2008).

Depression has effects on diverse processes within the brain, from monoamine metabolism (Oxenkrug 2010); to neurogenesis, neural survival, and neural plasticity (Lanni et al. 2009; Gass & Henn 2009); to dysfunctional neural circuitry (Caldecott-Hazard et al. 1988; Morris et al. 1999; Videbech 2000; Drevets et al. 2008); to endocrine responses (Pariante & Lightman 2008) and inflammatory cytokine release (Lanquillon et al. 2000; Maes et al. 1997). This has let to a large patchwork of hypotheses to explain depression, each with limitations. Various epidemiology studies have indicated both genetic and environmental components, with genetics contributing to susceptibility to depression (Kaufman et al. 2006; Caspi et al. 2003). While the

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genetic component of depression is generally accepted as involving many genes, few of those genes have been identified. A genetic dissection of the processes involved in depression requires the existence of animal models, of which there are relatively few. Using a pair of rat lines selectively bred for susceptibility and resistance to learned helplessness (Vollmayr & Henn 2001), I have been attempting to identify genes that play a role in depression.

History of treatment

A half century ago the prevailing view of the cause of depression was based on a psychodynamic view that the patient was turning unexpressed rage back towards the self. Such treatments featured many sessions over a span of years, with low effectiveness. In the 1960s Aaron T Beck introduced the cognitive model of depression based on observations during his practice. The origins of the model are reviewed here (Beck 2008). Briefly, he observed that his patients had thoughts that he termed automatic thoughts which affected how they interpreted events, viewed themselves, and their relationships with others. In depressed patients there was a consistent negative bias in their thoughts, resulting in the patients misinterpreting events or making erroneous assumptions. When he brought attention to these automatic thoughts and exposed the errors in thinking, the mood of the patients improved. Beck postulated that these errors, and the maladaptive beliefs on which they are based, are a core feature of depression. In the cognitive model, these belief and the resulting automatic thoughts re-enforce a despondent mood, generate negative attitudes, and influence behavior, which all feed back to re-enforce the beliefs. Since then a number of other psychotherapy methods have cropped up, however these are less relevant to the project at hand.

Towards the end of the 1950's the first antidepressant medications were developed and tested on people, such as imipramine (Azima 1959) and iproniazid (Loomer et al. 1957). These drugs affected monoamine signaling and metabolism,

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chiefly serotonin (Slattery et al. 2004). This led to the first biological approach to viewing and treating depression.

Phenotypic aspects of depression

Since the discovery of pharmacological interventions capable of improving the state of depressed patients, there is now ample evidence of a number of documented physiological effects on the brain and body as a result of depression. These include indications of increased immune system activation in depressed patients, as well as depression like symptoms in those experiencing chronic immune activation and alterations in the connections between the various limbic structures of brain. These competing hypotheses have served as the main framework from which researches have approached depression.

Phenotypic aspects of depression: The Monoamine Hypothesis

The responsiveness of patients to imipramine and iproniazid have led many researchers to explore the possibility that reduced serotonin activity within synapses were causative of depression. This monoamine hypothesis has been particularly popular in drug discovery research avenues, and today most drugs for treating depression have been developed based on this (Berton & Nestler 2006). There are a couple critical shortcomings; the acute effects of the drug activities occur within hours of drug administration, yet antidepressant effects are seen after at least two weeks of treatment. Thus the acute effects are not the direct cause of the antidepressant activity, instead, there appears to be via downstream effects that take longer to manifest and which researchers have only just begun to elucidate (Zazpe et al. 2007; Ampuero et al. 2010; Lee et al. 2010; Sun et al. 2012; Tsankova et al. 2006). Unfortunately, treatment resistance is a common problem in clinical situations, with 30% to 60% of patients exhibiting treatment resistance depending on the definition used (Amsterdam et al.

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1994; Fava 2003). These numbers include cases where medication loses effectiveness resulting after successful response to the drug.

Phenotypic aspects of depression: Inflammatory and Neurodegenerative Hypothesis

Observations of increased concentration of inflammatory cytokines in blood (Maes et al. 1997; Mikova et al. 2001) as well as the induction of depression due to treatment with interferon (Musselman et al. 2001) have led to the hypothesis that depression may be a result of immune system dysregulation (Miller et al. 2009). Furthermore immune system activation has been seen in response to social stress, via administration of a Trier Stress Test (Bierhaus et al. 2003).

Under immune system activation, the activity of indoleamine 2,3-dioxygenase (IDO) is increased, resulting in anhedoia and depression-like behaviors (O'Connor et al. 2009). IDO is the rate limiting enzyme in the production of kynurenines. The metabolites produced in this pathway include the NMDA agonist quinolinic acid which can cause excitotoxicity (Stone & Perkins 1981) and the cytotoxic 3-hydroxykynurenine (Eastman & Guilarte 1989). In consideration of this, it is not surprising that neurogenesis within the hippocampus is reduced in depressed and chronically stressed individuals (Gould et al. 1998).

In addition to neurogenesis, other aspects of neuroplasticity are involved in depression. Spine remodeling and dendritic branching are affected. Corticosterone injection in rats causes reductions in spine density and in the number of dendritic branches in the nucleus accumbens and hippocampus (Morales-Medina et al. 2009). The reduced spine density, which can result from either corticosterone injection or stress, can be mostly, but not completely reversed by antidepressant treatment as well as voluntary wheel running (Hajszan et al. 2009; Yau et al. 2011). Furthermore, administration of repeated electroconvulsive shock (ECS) increases the formation of serotonergic axons during regrowth of these axons following a hippocampal lesion (Madhav et al. 2000). While this does not show a role of axon sprouting in depression

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or the mechanism of electroconvulsive therapy (ECT), it appears to be the first evidence of ECS affecting this form of neuroplasticity.

Phenotypic Aspects of Depression: Neurocircuitry Hypothesis

The neurocircuitry based theories come from neuroanatomical data tracing connections between regions of the brain and are supported by imaging data, including fMRI and PET (Caldecott-Hazard et al. 1988; Morris et al. 1999; Strigo et al. 2008). These studies provide evidence of coordinated activity across various brain regions, and have indicated that activity within the limbic regions of affected individuals differ from unaffected individuals. Unfortunately, few treatments have been developed for this and some involve interventions far too invasive to be used except in the most intractable of cases (Sartorius et al. 2010).

The neurocircuitry of depression has however been an important context in which the others are approached, as most work is done focusing on specific regions of the brain identified as being involved in depression. One known circuit of particular importance is the hypothalamic-pituitary-adrenal (HPA) axis, which is hyperactive in depressed patients and is responsible for controlling glucocorticoid secretion (Pariante & Lightman 2008). The HPA axis provides a direct mechanism by which the neurocircuitry implicated in depression can trigger immune system activation via the pituitary, which secretes adrenocorticotrophic hormone (ACTH) triggering secretion of glucocorticoids by the adrenal glands. Indeed, many of these hypotheses include components that impact the pathways depicted in other hypotheses. Brain regions implicated by the neurocircuitry approaches to understanding depression have effects on central serotonin output via the dorsal raphe nuclei (DRN) (Amat et al. 2001). These effects can work together to strengthen the disease state and models have been used based on several of these hypotheses. Most of the monoamine systems, dopamine, serotonin, and norepinephrine, are under control of limbic regions that are implicated in the pathology of depression. The limbic system implicated in the neurocircuitry

approach outputs into the HPA axis via the pituitary. This allows depression to affect glucocorticoid release and regulate the immune system allowing the immune system to respond to stress. This may be beneficial during a classic fight or flight response where one is at risk for injury and exposure to pathogens. However in most everyday forms of stress, this is an overreaction with possible toxic consequences.

Learned Helplessness as a model for depression

Learned helplessness was first introduced in 1967 by J. Bruce Overmier and Martin E Seligman (Overmier & Seligman 1967). Initially discovered as an effect that interfered with Pavlovian conditioning, the authors ruled out some prevailing hypotheses for this effect and presented their own: that the inescapable nature of the shocks resulted in the animals learning that they were helpless. Their experiment was based on instrumental escape-avoidance training. Two chambers were divided by a barrier set to the height of the dog's shoulder. A light would go off and the dog was expected to jump the barrier. If the dog did not jump within 10 seconds, shock would be administered through the grid floor, and would terminate after the dog jumped the barrier or after 60 seconds. Naïve dogs learn within a few trials to jump the barrier when the light goes off and thus avoid shock. However, two thirds of dogs that had been previously immobilized in a hammock and subjected to shock via the footpads, failed to learn that barrier jumping avoids shock. Further research into this phenomenon has shown it is present not just in the dogs initially studied but in a wide range of animals, including human, and that the effect is not just a deficit in performing the shock avoidance test but poor performance in a range of adaptive behaviors (Willner 1991; McCann & Huhman 2012; Greenberg et al. 1989; Haracz et al. 1988). The important factor in this response has been controllability. Animals that were able to control the shock did not have the same escape deficits as those that could not.

Subsequent research has found that LH animals display anhedonia, as evidenced by preferences for sweet solutions in response to stress or increasing effort

required to access the rewarding drink (Vollmayr et al. 2004). Others have found sleep disturbances in the learned helpless rats that mimics the sleep disturbances present in depression (Adrien et al. 1991). Helpless rats have been observed to have a reduced ability to suppress corticosterone in response to dexamethasone treatment with no differences observed between naïve and non-helpless rats (Greenberg et al. 1989). This closely mirrors depression, where roughly half of patients are dexamethasone non-responders (Roy 1988).

Selective breeding for susceptibility and resistance to learned helplessness

Based on learned helplessness, F.A. Henn and colleagues began breeding rats based on outcomes in the learned helplessness test (Henn & Vollmayr 2005). Initially this was to create lines that would be a more consistent source of helpless or helpless resistant animals. To do this, they set up testing chambers that delivered electric shock through a grid floor, providing random shocks to the rat occupying the cage. Approximately 24 hours later those rats were placed in similar cages, but these were equipped with a lever. In these cages, shock would be delivered in a series of 15 trials with a maximum shock delivery of 60 seconds per trial and 15 seconds between trials. Pressing the lever will terminate a shock trial and a record is made of the time of all shock onsets, terminations, and lever presses. This procedure is described by Vollmayer and Henn (Vollmayr & Henn 2001). Each trial in which the rat terminated the shock within 20 seconds of onset was counted as a successful trial. Those that had less than five successes, were labeled learned helpless (LH); those that had more than ten successes were labeled helpless resistant, or non-learned helpless (NLH). Using this testing paradigm, Vollmayr and Henn bred two lines, one from each end of the spectrum. Starting with Sprague-Dawley rats, which score intermediate among rat lines with such tests, differences in the frequency of helpless animals were established after five generations of selective breeding (Henn & Vollmayr 2005). Their application of genetic isolation and selective pressure have yielded two distinct populations of rats, the

congenitally helpless (cLH) rats and the congenitally helpless resistant (cNLH) rats. The cLH rats have over a 90% frequency of scoring as learned helpless (LH) in the original test that developed the line; while the cNLH rats are resistant to developing learned helplessness. The breeding protocol they used started with five breeding pairs per line and avoided inbreeding, such that theoretically, genes that are related to susceptibility to depression will be enriched in alleles appropriate to the line while unrelated, unlinked loci should not be affected. However, with a small size of the breeding colonies and large number of generations of selective breeding, one can expect significant genetic homogeneity within the population.

These lines have been subjected to many studies to compare the model with depression. These include tests examining behaviors indicative of anhedonia showing a reduction in the motivation to acquire a pleasurable stimulus in the cLH line (Vengeliene et al. 2005; Vollmayr et al. 2004; Enkel et al. 2010). Analysis of open field behavior and the animals' responses to novelty show novelty induced hyperactivity in both lines with a sharp decrease indicating habituation to the environment (Schulz et al. 2009). Given the anhedonic aspects of the cLH line, this enhancement of the normal response is believed to be driven by anxiety rather than novelty-seeking. Additionally, fear conditioning and extinction tests show an increased fear response reduced fear extinction (Shumake et al. 2005) as would be expected in a depressive phenotype. These alterations in stress related aspects of cognition do not appear to be a result of a generalized deficit in learning as the selectively bred rats behave as well as the outbred Spague-Dawley rats in a Morris water maze (Vollmayr et al. 2004). There are also some intrinsic alterations in metabolic activity of several brain regions in the cLH rats demonstrated by cytochrome oxidase *in situ*s (Shumake et al. 2001; Shumake et al. 2004; Shumake et al. 2003; Shumake et al. 2002). This work demonstrated the central role of the lateral habenula (LHb) in helplessness (Li et al. 2011; Li et al. 2013).

In breeding these lines, Henn and colleagues have created a genetic model of depression that can be analyzed to find genes affecting susceptibility or resistance to developing depression in response to stress. More importantly, it may highlight how the

responses differ between a stress sensitive and stress resilient brain. Considering that twin studies have estimated a heritability between 25% and 64% (Kendler & Prescott 1999; Kendler et al. 1995; Ørstavik et al. 2007) in depression, it is likely that an individual's genetic makeup plays some role in determining whether an individual will develop depression.

In pursuit of the genetic variations that underlie the heritability observed in depression numerous genome-wide association studies have been performed. The first by Muglia et. al. in 2008 analyzes two sets of 1,022 patients versus 1,000 controls and 492 patients versus 1,052 controls and found no results that were statistically significant, either individually or in a meta-analysis of both samples together. The authors concluded that several thousand patients would be needed to get statistically significant results (Muglia et al. 2010). Several studies released soon after had similar sample sizes and similarly found no results reaching genome-wide significance standards (Sullivan et al. 2009; Shi et al. 2011; Shyn et al. 2011). Meta analyses performed on the data sets from the Sullivan et al., Shi et al., and Shyn et al. studies also failed to produce significant results despite a total sample size of nearly four thousand patients (Shyn et al. 2011). Another study on 2,431 patients also performed a meta analysis incorporating their work plus two additional studies, yet still found no associations with genome-wide significance (Wray et al. 2012).

There have been some successes, each producing only one or two significant associations. Two papers released in 2011 each found one gene. Kohli et al found a variant of Solute carrier protein family 6 member 15 (SLC6A15), while Aragam et al found a single nucleotide polymorphism (SNP) in adenylate cyclase activating polypeptide 1 receptor 1 (ADCYAP1R1) to be associated with depression (Kohli et al. 2011; Aragam et al. 2011).

The latest studies are meta-analyses. A large meta-analysis produced by the GWAS Consortium's Major Depressive Disorder Working Group performed an analysis of data sets containing over nine thousand patients and controls in the discovery phase and replication phase that consisted of 6,783 patients and 50,695 controls. The result

from analyzing such a massive data set was that the sample still does not have enough statistical power to resolve many of the genes involved in depression (GWAS Consortium 2012). With so much effort expended for such weak and scarce useable data, it is not surprising that researchers have attempted to look at the data in other way and to pursue genes with a suggestive level of statistical significance. A pathway analysis on the data set from Sullivan's work identified four pathways that were enriched in SNPs that were more common in depressed patients than in the non-depressed controls. These pathways were included those involved in neural function such as long-term depression and calcium signaling as well as several categories involved in cell adhesion and junctions between cells: cell adhesion molecules, arrhythmogenic right ventricular cardiomyopathy, and focal adhesion (Kao et al. 2012). Some researchers have examined the genes identified among the suggestive results via other means. Several SNPs in and around the piccolo (PCLO) gene were associated with depression in two GWAS studies (Sullivan et al. 2009; Aragam et al. 2011).

Genetic and environmental contributions to depression

As Barbara Vollmayr and Fritz Henn were establishing the selectively bred lines, others have published evidence that genetic variations contribute to susceptibility of depression (Lesch et al. 1996). Furthermore they found that for those with the susceptible genotype, the number of stressful life experiences correlated with scores on diagnostic tests for depression (Caspi et al. 2003). Also, this sub-population was more responsive to various risk factors, such as unavailability of social supports (Kaufman et al. 2004).

More recent work has shown that epigenetic changes at some of these genes correlate with number of stressful life experiences and that epigenetic marks are altered by antidepressant treatment (Murgatroyd et al. 2009; Wilkinson et al. 2009). The effects of the tricyclic antidepressant imipramine can be blocked by overexpression of histone deacetylase (HDAC) 5 in the hippocampus (Tsankova et al. 2006). This has led to

studies of sodium butyrate, an inhibitor of HDAC5, as a potential antidepressant (Schroeder et al. 2007). Additionally DNA methylation and demethylation have been implicated in processes ranging from activity dependent activation of neurogenesis to the long term adaptations of chronic intermittent ethanol exposure (Ma et al. 2009; Qiang et al. 2010). Such difficulties likely result from diverse etiologies, environmental effects, and a large number of genes that could be affecting susceptibility to developing depression. The reduced genetic and environmental diversity of the cLH and cNLH rats allow us to sidestep these problems in searching for genes which may play a role in the disorder.

Role of hippocampus in depression

   While many brain regions have been studied in relation to depression, one of the most extensively studied is the hippocampus. Evidence for a role in depression range from decreased hippocampal volume in depressed patients (Videbech & Ravnkilde 2004), to the effects of anti-depressants on animal models of depression (Zink et al. 2005), to changes in cell morphology related to neuronal plasticity (Morales-Medina et al. 2009; Hajszan et al. 2009), to PET imaging of cerebral blood flow that links hippocampal blood flow to Hamilton Depression scale scores (Videbech et al. 2002). Additionally, as one of two regions in which neuronal stem cells are know to produce new neurons even into adulthood (Kaplan & Bell 1984; Cameron et al. 1993; Kuhn et al. 1996), research looking at links between neurogenesis and depression measure the phenomenon in the hippocampus (Gould et al. 1998; Henn & Vollmayr 2004). Outputs from the hippocampus via the fornix connect to multiple structures important to the regulation of emotion including the hypothalamus and nucleus accumbens. Blocking hippocampal output via lesions of the fornix block the development of learned helplessness (Leshner & Segal 1979). We therefore judged it to be a good first target for expression studies, and focused our work on the changes in expression within the hippocampus of the congenitally helpless rats.

Chapter 2: Methods

To discover differentially expressed genes we performed expression arrays on cLH and cNLH animals trained and tested to ensure the subjects did expressed the helpless or helpless resistant phenotype. This setup would allow for detection of differentially expressed genes tied to either the trait of susceptibility versus resistance or the state of a learned helpless outcome versus more adaptive responses. The identified changes in expression could be something present regardless of treatment, a result of being helpless or non-helpless, or it could reflect a differential response to stress. The design chosen was one that could detect changes due to any of those three categories as we deemed this approach more useful even at the expense of the ability to differentiate between the categories.

Training and testing

All procedures were reviewed and approved by the BNL Institutional Animal Care and Use Committee (IACUC). Animals are housed in pairs, fed *ad libidum* and kept on a day-night cycle with 12 hours of light per day, starting at 7AM. Fifteen week old rats are trained during the morning hours between 8am and 1pm. Outbred control Sprague-Dawley (SD) rats were ordered from Taconic and were allowed to acclimate to the animal facility for at least two weeks before using in experiments.

The training consists of a 40 minute session during which the rats are subjected to brief foot shocks with a pseudo-random intervals. The next day, rats are placed in cages equipped with levers and a cue light for the testing session. The testing session consists of 15 trials. Each trial starts onset of foot-shock and lasts either until the lever is pressed or one minute elapses. The cue light illuminates the lever while shock is being delivered. The trials are separated by 15 second intervals of no shock. Animals

that terminate shock by pressing the lever on fewer than five trials are considered helpless (LH). Those that terminate shock within twenty seconds of shock onset by pressing the lever on more than ten trials are considered helpless resistant or non-learned helpless (NLH). The naive rats were not subjected to either training or testing so as to serve as non-stressed controls.

RNA extraction

Tissue is harvested from animals four days after the test day. For the microarrays and qPCR, rats are sacrificed by decapitation followed immediately by dissection and freezing of hippocampal tissue in liquid nitrogen.

Total RNA was extracted from rat hippocampal tissue by homogenizing the frozen tissue directly into 2mL of TRIreagent (Molecular Research) in Tenbroock 5mL homogenizers. After addition of 0.4mL of chloroform and centrifugation, 0.75mL of the aqueous phase was removed to a new tube and isopropanol precipitated. After three washes in 70% ethanol, the RNA was resuspended in 20µL DEPC treated water (Ambion), concentration was determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific).

cDNA synthesis

For microarrays, the cDNA was produced using Invitrogen's Superscript Double-Stranded cDNA Synthesis Kit, using 20µg to 50µg of RNA in a reaction volume of 20µL for first strand synthesis and 150µL for the second strand synthesis. Each reaction is run overnight at 45°C for first strand synthesis, and 16°C for the second strand synthesis. At the end of the second reaction, 2µL of T4 Polymerase are added and incubation continued 10 minutes before adding 10µL 0.5M EDTA then 4µg RNase A. The RNase A is allowed to digest at 37°C for 15 minutes before phenol:chloroform extraction and ethanol precipitation. The product is then checked on the Nanodrop and

on an agarose gel for concentration and quality.

For quantitative PCR, we used the QuantiTect Reverse Transcription Kit (QIAgen). Purified RNA was treated with DNase I (10µL purified RNA, 2.5µL 2.8U/µL RNase-free-DNase I, 5µL 10x Buffer RDD, 22.5µL DEPC-treated water). After one hour at 37°C, the reaction was phenol:chloroform extracted, using TRIreagent in place of phenol, then isopropanol precipitated, washed twice with 75% ethanol, and resuspended in 20µL RNase free water. The DNase treated RNA was then quantified on Nanodrop and diluted to a concentration of 1µg/µL. 2µg of RNA was then used in the Quantitect Reverse Transcription Kit as detailed in their manual. Briefly, the RNA was incubated 2 minutes at 42°C in their genomic DNA Wipeout buffer, before returning to where the RT buffer, primer mix and reverse transcriptase is added. Then the reaction is incubated 15 minutes at 42°C followed by inactivation at 95°C for 3 minutes.

Microarray

We used Nimblegen's Rat expression 385K arrays (design: 2007-07-20_Rat_X1_expr). This array contains 373,400 probes, 14 probes each for 26,739 genes, with probes randomly distributed throughout the array. Double-stranded cDNAs generated from total RNA as detailed above were shipped to Roche Nimblegen. There, Nimblegen personnel performed quality control, generated labeled cDNA and hybridized the labeled cDNA to the expression arrays. Within each array, calls for each gene were determined by averaging the 14 probes for that gene, with any outliers being dropped, and the average calculated on the remaining probes. Data was normalized using Robust Multi-chip Averaging (RMA) and sent back to us for further analysis.

qPCR

We used the Rotor-Gene SYBR Green PCR Kit in the Rotor-Gene (machine name)(QIAgen). The primers for protocadherin gamma A 11 (Pcdhga11) were

Pcdhga11-398F: 5'-TAG GAT CGA TCG TGA AGA GC-3' and Pcdhga11-500R: 5'-TTC CAC CTC CAC TCC ATA AA-3'. Gapdh served as the control with the primers Gapdh943F: 5'-TTC TTC CAC CTT TGA TGC TG-3' and Gapdh1062R: 5'-ATG TAG GCC ATG AGG TCC AC-3'. The products of the Quantitect Reverse Transcription reactions were diluted in a series of five fivefold dilutions, with 2μL of each dilution used in the reactions, with three replicates per combination of dilution, primer, and sample. Three rats of each condition were tested.

Western blot

Rat hippocampus were homogenized in ice cold 150μL RIPA lysis buffer with protease inhibitors added as recommended by the manufacturer(Santa Cruz Biotechnology, Inc.), and the volume was increased to 1mL with more RIPA then vortexed at 4°C for 30 minutes. As much of the membranes remained resistant to solublization with just the NP40 in RIPA, SDS and β-mercaptoethanol were added to 1% and 10mM respectively. The lysate was boiled 5 minutes at 90°C. Yield was estimated by BCA assay (Pierce) on a 96-well microplate and read with the Bio-Rad model 680 microplate reader. This was done using an aloquot reserved from the lysate after addition of SDS but before addition of β-mercaptoethanol. Several quantities of protein were boiled in SDS-PAGE loading buffer and separated on 7.5% polyacrylamide gel. Gels were then transferred onto PVDF membranes (Bio-Rad).

Blots were blocked for one hour in Tris buffered saline + 0.25% Tween-20 (TBS-T) + 10% bovine serum albumin (BSA), then incubated in primary antibody overnight at 4°C in TBS-T+5%BSA. After primary, the blots are washed four times in TBS-T, then incubated one hour at room temperature in secondary antibody in TBS-T+5%BSA. After four more washes in TBS-T, detection is performed by addition of enhanced chemiluminescence substrate mixture (ECL Plus, GE Healthcare; Santa Cruz Biotechnologies; 1:5000) exposure and development of film, which is then scanned with a flatbed scanner. Protein levels were quantified in arbitrary units (A.U.) after actin

normalization using NIH ImageJ. Antibodies and concentrations used are goat anti-pcdhga11 (E-14) at 1:400, mouse anti-b-actin at 1:8000, anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies at 1:8000.

Fixation of rat brains by vascular perfusion

Rats were perfused with PBS + 4% formaldehyde (from paraformaldehyde) via a cannula inserted into the heart. Rats were first anesthetized with 75mg/kg ketamine plus 0.5mg/kg Domitor (medetomidine). When no longer responsive, the rat was laid supine, and the skin was cut away from the chest and abdomen. A cut was made across the peritoneal wall and extended up either side to allow exposure of the diaphragm. Following the cutting of the diaphragm, we used scissors to cut through the ribs on either side of the chest and reflected the ribs and sternum up to expose the heart, clamping the ribs in this position. Any fat around the heart was cleared away and the protrusion of the left ventricle was cut with scissors to thin the ventricle wall for cannula insertion. The perfusion pump is filled with PBS and set to a slow drip then inserted into the heart with the end of the cannula projecting into the aorta 1-3mm. A cut is made in the right atrium and the flow rate is increased to flush the blood from the body. Once the fluid coming out of the right atrium is clear, switch to 4% formaldehyde (paraformaldehyde derived) in PBS and pump 300-350mL of the fixative through the rat.

Immediately following perfusion, the brain is removed and placed in scintillation vials containing PBS + 4% formaldehyde and incubated overnight at 4°C. After the overnight incubation, the formaldehyde solution is replaced with PBS + 30% sucrose and stored at 4°C.

Tissue culture of rat hippocampal tissue

Tissue was dissociated in 2.5mg/mL papain, 2.5mg/mL trypsin in Hanks Balanced Salt Solution for 25 minutes with gentle mixing. The cells were then washed

twice in Neurobasal Media (GIBCO/Life Technologies) with 2% fetal bovine serum (FBS), then resuspended and cells were counted dispensed into wells containing coverslips coated with poly-L-lysine and fibronectin. The cells were incubated 30 minutes at 37°C to allow attachment to the coverslips, after which additional media was added and the cells were cultured for 16 days with regular feedings of Neurobasal Media with 2% FBS, 10ng/mL BDNF, and 10ng/mL NT3 prior to fixation and immunolabeling.

Immunofluorescence

Fixed brain tissue was mounted to the microtome stage by freezing in OCT medium using dry ice/methanol. The tissue was kept frozen on dry ice/methanol while 40μm coronal sections were cut and placed in PBS for storage.

Selected sections were probed with various combinations of the following antibodies: goat anti-pcdhga11 (A-12) (Santa Cruz Biotechnology, Inc.) used at 1:50, mouse anti-NeuN (Millipore) used at 1:100, rabbit anti-glial fibrilary acidic protein (GFAP) (Millipore) used at 1:1000, mouse anti-microtubule associated protein 2 (MAP-2) (Santa Cruz Biotechnology, Inc.) used at 1:50. The secondary antibodies used were Alexa 647 conjugated donkey anti-mouse (Invitrogen) used at 1:10,000; and Cy3 conjugated anti-rabbit and anti-mouse (Novus Biologicals) used at 1:750.

Sections were blocked in phosphate buffered saline + 0.25% Triton X-100 (PBS-T) + 10% normal donkey serum (NDS) for one hour at room temperature, then incubated in primary at the above mentioned dilutions in PBS-T + 5% NDS overnight at 4°C. Sections were washed four times in PBS-T before incubation in secondary for 2-4 hours at room temperature at the above mentioned dilutions in PBS-T + 5% NDS. The sections were then washed five times in PBS-T before being mounted on gelatin subbed slides, air dried, and mounted under coverslips with MOWIOL mounting medium, with the edges sealed by nail polish.

Slides are visualized on a Leica DMI 6000 Inverted Confocal microscope using a TCS-SP5 scanner connected to a computer loaded with the Leica Application Suite Advanced Fluorescence (LAS AF) software package. Excitation was with HeNe 543nm laser and HeNe 633nm laser. Emission filters TRITC 595/50 and Cy5 700/100. Stacks of six to twelve optical sections approximately 0.6µm in thickness were collected across a depth of 3.0µm to 3.5µm and with the LAS AF software and assembled into z-projections using NIH ImageJ. The projections were then imported into GNU Image Manipulation Program (GIMP ver.2.8.4 http://www.gimp.org/) where they were assembled into a panoramic of the entire hippocampal section. The default color of the Cy5 channel was switched to green for improved visibility and a minimal correction for improved contrast was applied (Menu->Colors->Levels: Green channel set to 20/1.00/255; Red channel set to 13/1.00/200; Black Point/Gamma/White Point) uniformly to all images. The resulting image was then subjected to 1 pixel radius Gaussian blur. Full panoramic was scaled to 10% of original size for inclusion in this document while insets were cropped from the full size image. Original unenhanced and major intermediates available as requested.

Chapter 3: Microarray of Hippocampal Tissue

We trained and tested sufficient number of wild type rats to acquire five Sprague-Dawley LH and four Sprague-Dawley NLH specimens, harvesting and freezing in liquid nitrogen bilateral whole hippocampus from these and six naive rats. Also we trained and tested sufficient cLH and cNLH to generate five each of cLH testing LH and cNLH testing NLH; all of these were rapidly dissected to isolate and freeze both right and left hippocampi. The hippocampal tissue of the rats were used to create total RNA extracts using TRIreagent. The RNA was used to create double stranded cDNA via the Superscript II kit (Invitrogen). The resulting cDNA was subjected to phenol:chloroform extraction and a check for concentration and quality before sending cDNA to Roche-Nimblegen. There, the cDNA was used to create labeled probes which were then used in the microarray. The data was filtered based on several analyses to produce various lists. An ANOVA of all five conditions was filtered based on a two fold change between either cLH or cNLH versus naive. This resulted in a list of 230 genes, 48 of which had statistical significance of 98% or above (Table 1). When classified by helplessness outcome none of the 76 genes with two fold changes in expression had any degree of statistical significance (data not shown). A comparison of outbred Sprague-Dawley animals ordered from Taconic and those selectively bred at our animal facility produced 70 genes with both a two fold change in expression and a statistical significance of 98% or better (Table 2). Many of the top results from the initial ANOVA were among the genes in this set.

With so many genes indicating breeding effects, we switched to a direct analysis of cLH versus cNLH. Comparing the selectively bred lines using a moderated T-test and Benjamini Hochberg false discovery rate correction, we found thirteen genes showing two-fold or greater change in expression within a 95% confidence interval, five of which have a greater than 99% confidence. These transcripts are listed in Table 3 by

P-value, with most significant listed first. Many of the top genes on the list relate to pathways believed to be subject to dysregulation in depression. These include a member of the kyenurenine metabolic pathway, a solute carrier protein that when mutated causes a failure to form serotonin containing platelet dense granules, and a gene involved in the cGMP-dependant protein kinase pathway.

Table 1: (Following page) List of genes with two-fold change in expression between either selectively bred line and naïve wild-type (Sprague-Dawley) animals. List is sorted by P-value from a one way ANOVA.

SEQ_ID	Updated Accession Number	Transcript Name	Gene Symbol	Naive	WT LH	WT NLH	cLH	cNLH	Fold Change (cLH v Naive)	Fold Change (cNLH v Naive)	Fold Change (cLH v cNLH)	P value (ANOVA)	P value (cLH v cNLH)
NM_022936		epoxide hydrolase 2, cytoplasmic	Ephx2	1667	2155	1855	208	236	8.00 -fold down	7.06 -fold down	1.13 -fold down	1.58E-008	1
XM_001058570	Record Removed	similar to cytochrome P450, family 2, subfamily J, polypeptide 4 (predicted)	RGD1560981_predicted	30	30	36	235	242	7.76 -fold up	8.00 -fold up	1.03 -fold down	9.45E-008	1
NM_203410		interferon, alpha-inducible protein 27	Ifi27	8677	8911	9339	1985	2448	4.37 -fold down	3.54 -fold down	1.23 -fold down	6.40E-007	1
NM_001039345		equatorin, sperm acrosome associated	Eqtn	39	34	34	137	129	3.51 -fold up	3.32 -fold up	1.06 -fold up	8.24E-007	1
NM_053781		aldo-keto reductase family 1, member B7	Akr1b7	50	56	46	166	186	3.28 -fold up	3.69 -fold up	1.13 -fold down	1.27E-006	1
XM_001070453	NM_001107118	ring finger protein (C3H2C3 type) 6	Rnf6	3316	3208	3452	819	615	4.05 -fold down	5.39 -fold down	1.33 -fold up	1.27E-006	1
NM_173124		cytochrome P450, family 4, subfamily f, polypeptide 5	Cyp4f5	294	399	355	40	18	7.31 -fold down	16.53 -fold down	2.26 -fold up	2.30E-006	1
NM_172223		peroxisomal membrane protein 4	Pxmp4	359	353	513	1415	1504	3.94 -fold up	4.19 -fold up	1.06 -fold down	3.49E-006	1
XM_001062650		transmembrane protein 44 (transcript variant X1) (predicted)	Tmem44	1339	1459	1623	401	494	3.34 -fold down	2.71 -fold down	1.23 -fold down	3.72E-006	1
NM_001033704		xylulokinase homolog (H. influenzae)	Xylb	204	190	239	632	668	3.09 -fold up	3.27 -fold up	1.06 -fold down	1.28E-005	1
NM_147207	*	ischemia related factor vof-16	Vof16	662	581	675	229	196	2.89 -fold down	3.38 -fold down	1.17 -fold up	2.26E-005	1
XM_001070425	Record Removed	Jun D proto-oncogene (predicted)	Jund	182	153	195	490	491	2.70 -fold up	2.70 -fold up	1.00 -fold up	2.84E-005	1
XM_001066248	Record Removed	coiled-coil domain containing 150 (predicted)	Ccdc150	176	185	190	68	64	2.60 -fold down	2.73 -fold down	1.05 -fold up	5.80E-005	1
XM_001077664	NM_001271352	cytochrome P450, family 4, subfamily f, polypeptide 37	Cyp4f37	287	354	341	98	77	2.94 -fold down	3.75 -fold down	1.27 -fold up	6.03E-005	1
XM_001065432	Record Removed	hypothetical protein LOC683326 (predicted)	LOC683326	85	80	95	319	276	3.75 -fold up	3.24 -fold up	1.16 -fold up	8.43E-005	1
XM_001078027	Record Removed	similar to Cytochrome P450 4F5 (predicted)	CYPIVF5	275	308	313	74	58	3.71 -fold down	4.74 -fold down	1.28 -fold up	1.59E-004	1
NM_001044265		STEAP family member 4	Steap4	83	103	107	163	252	1.97 -fold up	3.04 -fold up	1.54 -fold down	3.17E-004	0.635
NM_001034028		hypoxia up-regulated 1 (transcript variant 2)	Hyou1	9581	5893	9613	1605	1112	5.97 -fold down	8.61 -fold down	1.44 -fold up	4.40E-004	1
NM_001014125		protein disulfide isomerase family A, member 5	Pdia5	215	244	215	622	635	2.90 -fold up	2.96 -fold up	1.02 -fold down	5.92E-004	1
XM_224732	Record Removed	regulator of nonsense transcripts 1 (predicted)	Rent1_predicted	400	427	486	898	779	2.24 -fold up	1.95 -fold up	1.15 -fold up	6.10E-004	1
NM_198744		tumor protein D52-like 2	Tpd52l2	2092	2283	2041	871	464	2.40 -fold down	4.51 -fold down	1.88 -fold up	7.15E-004	1
NM_138875		Jun D proto-oncogene (transcript variant 1)	Jund	501	499	615	1012	1024	2.02 -fold up	2.04 -fold up	1.01 -fold down	8.71E-004	1
XM_001065561	NM_001106707†	NLR family, CARD domain containing 4	Nlrc4	50	70	60	111	122	2.24 -fold up	2.45 -fold up	1.10 -fold down	0.0011	1
XM_001076821	NM_001108069	serine/threonine kinase 11	Stk11	774	418	770	2070	1834	2.67 -fold up	2.37 -fold up	1.13 -fold up	0.0013	1
NM_031800		death effector domain-containing	Dedd	373	272	352	763	836	2.05 -fold up	2.24 -fold up	1.10 -fold down	0.0015	1
NM_001008560		protease, serine, 35	Prss35	288	552	835	2195	2192	7.62 -fold up	7.61 -fold up	1.00 -fold up	0.0015	1
XM_001069582	NM_001106077	MAU2 sister chromatid cohesion factor (transcript variant 1)	Mau2	1807	1870	1854	3468	3671	1.92 -fold up	2.03 -fold up	1.06 -fold down	0.0024	1
XM_001058809	Record Removed	similar to cellular repressor of E1A-stimulated genes 2 (predicted)	RGD1564056_predicted	487	262	524	1233	1129	2.53 -fold up	2.32 -fold up	1.09 -fold up	0.0029	1
XM_001081491	NM_001107052	ADP-ribosylation factor-like 4D	Arl4d	378	614	461	74	69	5.08 -fold down	5.50 -fold down	1.08 -fold up	0.0031	1
XM_001068074	NM_001106460	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	Ptpn22	83	91	81	128	200	1.55 -fold up	2.41 -fold up	1.56 -fold down	0.0037	1
XM_001073069	NM_001107522	solute carrier family 35, member D3	Slc35d3	153	149	144	74	181	2.05 -fold down	1.19 -fold up	2.43 -fold down	0.0052	0.0129
NM_001008850		RT1 class I, locus M1, gene 4	RT1-M1-4	220	256	346	45	50	4.89 -fold down	4.43 -fold down	1.10 -fold down	0.0053	1
XM_001060531	Record Removed	serum/glucocorticoid regulated kinase 3 (transcript variant 1) (predicted)	Sgk3	276	174	249	1017	873	3.68 -fold up	3.16 -fold up	1.16 -fold up	0.0057	1
NM_021858		guanine nucleotide binding protein (G protein), beta polypeptide 3	Gnb3	363	462	392	94	178	3.86 -fold down	2.04 -fold down	1.89 -fold down	0.0061	1
NM_020096		interferon-induced protein with tetratricopeptide repeats 1	Ifit1	45	38	49	47	959	1.05 -fold up	21.23 -fold up	20.30 -fold down	0.0071	0.334
XM_001078563	Record Removed	patatin-like phospholipase domain containing 1 (predicted)	Pnpla1	491	625	406	2479	2538	5.05 -fold up	5.17 -fold up	1.02 -fold down	0.0077	1
NM_153467		protein phosphatase 1, regulatory subunit 17 (a.k.a. G substrate)	Ppp1r17 (Gsbs)	149	129	131	332	97	2.22 -fold up	1.55 -fold down	3.44 -fold up	0.0078	0.0135
NM_012872		phosducin	Pdc	50	43	50	145	134	2.89 -fold up	2.66 -fold up	1.09 -fold up	0.0079	1
XM_001058776		transmembrane epididymal protein 1-like (predicted)	RGD1560559	29	30	29	113	118	3.88 -fold up	4.06 -fold up	1.04 -fold up	0.0096	1
XM_227485	NM_001107702	Fc receptor-like 2	Fcrl2	830	825	797	122	150	6.83 -fold down	5.53 -fold down	1.24 -fold down	0.0097	1
XM_001074225	NM_001173449	camello-like 2	Cml2	281	286	322	110	111	2.55 -fold down	2.53 -fold down	1.01 -fold down	0.0098	1
NM_001029920		V-set and transmembrane domain containing 4	Vstm4	236	335	289	476	346	2.02 -fold up	1.47 -fold up	1.38 -fold up	0.016	1
XM_001079217	NM_001107449	chloride channel accessory 1	Clca1	773	991	702	260	486	2.98 -fold down	1.59 -fold down	1.87 -fold down	0.017	0.606
XM_222076		scavenger receptor cysteine rich domain containing, group B (4 domains) (transcript variant X2)	Srcrb4d	227	280	159	100	119	2.26 -fold down	1.91 -fold down	1.18 -fold down	0.018	1
XM_001072344		histone H4 variant H4-v.1 (predicted)	RGD1562378	223	232	195	488	472	2.19 -fold up	2.12 -fold up	1.03 -fold up	0.018	1
NM_001001972		lymphocyte antigen 6 complex, locus G6E	Ly6g6e	147	197	158	76	64	1.94 -fold down	2.28 -fold down	1.18 -fold up	0.018	1
XM_001059035	Record Removed	isthmin-2-like (predicted)	LOC681936	169	167	194	256	361	1.52 -fold up	2.14 -fold up	1.41 -fold up	0.018	1
NM_175756		Fc fragment of IgG, low affinity IIb, receptor (CD32)	Fcgr2b	488	527	446	181	214	2.70 -fold down	2.28 -fold down	1.19 -fold down	0.019	1

*: This RefSeq was permanently suppressed because it is now thought that this gene does not encode a protein.

†: NM_001106707.1: This RefSeq was suppressed temporarily based on the calculation that the encoded protein was shorter than proteins from the putative ortholog from human: NLRC4 (GeneID:58484).

SEQ_ID	Updated Accession Number	Transcript	Gene Symbol	Taconic (WT)	Selectiely bred	Fold Change	P value
NM_022936		epoxide hydrolase 2, cytoplasmic	Ephx2	1798	220	8.16 -fold down	0
XM_001058570	Record Removed	similar to cytochrome P450, family 2, subfamily J, polypeptide 4 (predicted)	RGD1560981_predicted	31	232	7.56 -fold up	0
NM_001039345		equatorin, sperm acrosome associated	Eqtn	35	130	3.67 -fold up	3.86E-011
NM_203410		interferon, alpha-inducible protein 27	Ifi27	8669	2194	3.95 -fold down	3.96E-011
XM_001070453	NM_001107118	ring finger protein (C3H2C3 type) 6	Rnf6	3258	677	4.81 -fold down	5.82E-011
NM_053781		aldo-keto reductase family 1, member B7	Akr1b7	50	173	3.45 -fold up	1.22E-010
NM_173124		cytochrome P450, family 4, subfamily f, polypeptide 5	Cyp4f5	325	23	14.03 -fold down	1.84E-010
NM_172223		peroxisomal membrane protein 4	Pxmp4	381	1440	3.78 -fold up	1.1E-009
XM_001062650		transmembrane protein 44 (transcript variant X1) (predicted)	Tmem44	1415	440	3.21 -fold down	1.36E-009
NM_001033704		xylulokinase homolog (H. influenzae)	Xylb	203	638	3.14 -fold up	1.93E-009
NM_147207		ischemia related factor vof-16	Vof16	630	204	3.09 -fold down	3.73E-009
XM_001070425	Record Removed	Jun D proto-oncogene (predicted)	Jund	171	485	2.84 -fold up	1.12E-008
XM_001066248	Record Removed	coiled-coil domain containing 150 (predicted)	Ccdc150	178	65	2.72 -fold down	1.16E-008
XM_001065432	Record Removed	hypothetical protein LOC683326 (predicted)	LOC683326	82	292	3.57 -fold up	1.28E-008
XM_001077664	NM_001271352	cytochrome P450, family 4, subfamily f, polypeptide 37	Cyp4f37	309	85	3.66 -fold down	1.64E-008
XM_001078027	Record Removed	similar to Cytochrome P450 4F5 (predicted)	CYPIVF5	281	63	4.5 -fold down	0.000000028
NM_001014125		protein disulfide isomerase family A, member 5	Pdia5	216	620	2.88 -fold up	0.000000182
NM_198744		tumor protein D52-like 2	Tpd52l2	2122	566	3.75 -fold down	0.000000656
XM_001078563	Record Removed	patatin-like phospholipase domain containing 1 (predicted)	Pnpla1	397	2382	6 -fold up	0.00000361
NM_001034028		hypoxia up-regulated 1 (transcript variant 2)	Hyou1	7433	1294	5.75 -fold down	0.00000362
NM_001034946		intelectin 1 (galactofuranose binding)	Itln1	90	200	2.22 -fold up	0.00000448
NM_001008850		RT1 class I, locus M1, gene 4	RT1-M1-4	229	46	4.95 -fold down	0.00000453
XM_227485	NM_001107702	Fc receptor-like 2	Fcrl2	685	133	5.15 -fold down	0.00000573
NM_012872		phosducin	Pdc	45	133	2.95 -fold up	0.00000596
NM_031800		death effector domain-containing	Dedd	326	768	2.36 -fold up	0.00000653
XM_001058776		transmembrane epididymal protein 1-like (predicted)	RGD1560559	28	100	3.52 -fold up	0.00000697
XM_001060531	Record Removed	serum/glucocorticoid regulated kinase 3 (transcript variant 1) (predicted)	Sgk3	205	893	4.35 -fold up	0.00000702
NM_001008560		protease, serine, 35	Prss35	397	2167	5.45 -fold up	0.0000078

SEQ_ID	Updated Accession Number	Transcript	Gene Symbol	Taconic (WT)	Selectiely bred	Fold Change	P value
XM_001074225	NM_001173449	camello-like 2	Cml2	280	106	2.65 -fold down	0.0000081
XM_001076821	NM_001108069	serine/threonine kinase 11	Stk11	586	1907	3.26 -fold up	0.00000985
XM_001081491	NM_001107052	ADP-ribosylation factor-like 4D	Arl4d	399	71	5.63 -fold down	0.0000102
XM_001067252	NM_001107702	Fc receptor-like 2	Fcrl2	658	133	4.96 -fold down	0.0000185
XM_001072344		histone H4 variant H4-v.1 (predicted)	RGD1562378	206	477	2.32 -fold up	0.0000276
XM_001055606		oxysterol binding protein-like 3 (transcript variant X1)	Osbpl3	74	226	3.04 -fold up	0.0000297
NM_001008849		RT1 class I, locus M1, gene 2	RT1-M1-2	667	109	6.12 -fold down	0.0000304
NM_001044265		STEAP family member 4	Steap4	94	200	2.14 -fold up	0.0000344
NM_021858		guanine nucleotide binding protein (G protein), beta polypeptide 3	Gnb3	385	119	3.22 -fold down	0.0000344
NM_175756		Fc fragment of IgG, low affinity IIb, receptor (CD32)	Fcgr2b	465	191	2.44 -fold down	0.0000413
NM_001001972		lymphocyte antigen 6 complex, locus G6E	Ly6g6e	162	64	2.56 -fold down	0.0000805
XM_001058809	Record Removed	similar to cellular repressor of E1A-stimulated genes 2 (predicted)	RGD1564056_predicted	373	1153	3.09 -fold up	0.000129
NM_022617		macrophage expressed gene 1	Mpeg1	2800	539	5.19 -fold down	0.000184
XM_001058415	Record Removed	desmoplakin, transcript variant 1	Dsp	1243	3189	2.57 -fold up	0.000276
XM_001072499	Record Removed	similar to germinal histone H4 gene	RGD1565073	210	440	2.1 -fold up	0.00031
NM_021668		camello-like 1	Cml1	96	239	2.5 -fold up	0.000464
XM_001066387		serine (or cysteine) peptidase inhibitor, clade B, member 1b	Serpinb1b	67	171	2.56 -fold up	0.000784
NM_001000806		olfactory receptor 1250	Olr1250	59	135	2.29 -fold up	0.00104
XM_001060603	NM_001107175	protein phosphatase 1, regulatory subunit 15B	Ppp1r15b	316	737	2.33 -fold up	0.00139
XM_001054531	Record Removed	similar to ADAM 21 precursor (A disintegrin and metalloproteinase domain 21) (ADAM 31)	LOC679062	59	124	2.12 -fold up	0.00159
XM_001079816	NM_001107596	transmembrane protein 2	Tmem2	343	1127	3.29 -fold up	0.00159
NM_001013913		solute carrier family 16, member 4	Slc16a4	96	192	2 -fold up	0.00181
XM_001060466	NM_001105951	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta	Pik3c2b	911	327	2.78 -fold down	0.00218
XM_001076373	Record Removed	cadherin-related family member 3-like	LOC686932	101	222	2.2 -fold up	0.00253
XM_001079217	NM_001107449	chloride channel accessory 1	Clca1	776	345	2.25 -fold down	0.00327
XM_578421	Record Removed	RGD1560270 (predicted)	RGD1560270_predicted	860	331	2.6 -fold down	0.00344
XM_001068195	NM_001106075	kelch-like family member 26	Klhl26	349	158	2.2 -fold down	0.00364
NM_139090		activin A receptor, type IC	Acvr1c	1461	3160	2.16 -fold up	0.00371

SEQ_ID	Updated Accession Number	Transcript	Gene Symbol	Taconic (WT)	Selectiely bred	Fold Change	P value
XM_001080306	NM_001106249	sialic acid binding Ig-like lectin 5	Siglec5	697	1562	2.24-fold up	0.00391
XM_001054923	Record Removed	CD84 molecule	Cd84	206	478	2.32-fold up	0.00409
XM_001075648	NM_001108730	major facilitator superfamily domain containing 12	Mfsd12	277	129	2.15-fold down	0.00435
NM_001003959		DNA (cytosine-5-)-methyltransferase 3 beta	Dnmt3b	61	131	2.15-fold up	0.00496
NM_173325	*	putative retrovirus-related gag protein	LOC286991	466	166	2.8-fold down	0.00674
NM_030853		linker for activation of T cells	Lat	184	371	2.02-fold up	0.00685
NM_001034011		SFT2 domain containing 2	Sft2d2	170	374	2.2-fold up	0.00701
XM_001058849	Record Removed	similar to death effector domain-containing (predicted)	RGD1563412_predicted	155	384	2.48-fold up	0.00728
XM_001064542	NM_001114602	protocadherin beta 5	Pcdhb5	1098	2711	2.47-fold up	0.00761
XM_001055177	Record Removed	protocadherin beta 5 (predicted)	Pcdhb5_predicted	1054	2682	2.54-fold up	0.00801
XM_001079643		coiled-coil domain containing 73 (transcript variant X1)	Ccdc73	122	251	2.06-fold up	0.00923
XM_001080963		ATPase family, AAA domain containing 5 (transcript variant X1)	Atad5	191	391	2.05-fold up	0.0103
XM_001077062	Record Removed	similar to collagen, type XXIV, alpha 1	LOC687191	218	462	2.12-fold up	0.0139
XM_001061821	Record Removed	DNA methyltransferase 3B (transcript variant 3)	Dnmt3b	44	89	2.04-fold up	0.0143

*: NM_173325.1: This RefSeq was permanently suppressed because it is based on sequence from an endogenous retrovirus.

Table 2: (This page and preceding two pages) List of genes with two-fold difference between the selectively bred lines and the wild-type (Sprague-Dawley) animals ordered from Taconic. Entries are sorted by P value from a moderated t-test between wild type (all outcomes) v. selectively bred (cLH and cNLH)

SEQ_ID	Updated Accession Number	Transcript Name	Gene symbol	Naive	WT LH	WT NLH	cLH	cNLH	Fold Change (cLH v cNLH)	P value
NM_001013086		capping protein (actin filament), gelsolin-like	CapG	179	207	207	112	260	2.33 -fold down	0.00721
NM_021593		kynurenine 3-monooxygenase	Kmo	29	43	35	39	119	3.06 -fold down	0.0073
NM_013167		uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	82	106	82	186	44	4.21 -fold up	0.00821
XM_001072442	Record removed	similar to Alpha-fetoprotein precursor (Alpha-fetoglobulin) (Alpha-1-fetoprotein)	LOC684913	71	80	43	36	135	3.72 -fold down	0.00891
NM_001037140		protocadherin gamma subfamily A, 1	Pcdhga1	259	257	242	91	420	4.62 -fold down	0.00902
XM_001077986	NM_001135259	COX assembly mitochondrial protein 1	Cmc1	491	704	484	1704	574	2.97 -fold up	0.0114
NM_001037153		protocadherin gamma subfamily A, 11	Pcdhga11	638	741	924	1261	485	2.60 -fold up	0.0126
XM_001073069	NM_001107522	solute carrier family 35, member D3	Slc35d3	153	149	144	74	181	2.43 -fold down	0.0129
NM_153467		protein phosphatase 1, regulatory subunit 17 (G-substrate)	Ppp1r17, Gsbs	149	129	131	332	97	3.44 -fold up	0.0135
XM_001062594	NM_001115044	similar to paired immunoglobin-like type 2 receptor beta	LOC685157	74	66	37	57	262	4.63 -fold down	0.0275
XM_001060704	NM_001107704*	chromodomain helicase DNA binding protein 1-like	Chd1l	547	660	623	360	757	2.10 -fold down	0.0278
XM_001066237	NM_001106453	tripartite motif-containing 45	Trim45	476	626	392	241	741	3.07 -fold down	0.0291
XM_001076997		glutamate decarboxylase-like 1	Gadl1	67	74	54	45	116	2.55 -fold down	0.0307

*: NM_001107704.1: This RefSeq was suppressed temporarily based on the calculation that the encoded protein was shorter than proteins from the putative ortholog from zebrafish: chd1l (GeneID:393283).

Table 3: List of genes with two-fold or greater change in expression between cLH and cNLH lines. List sorted by P value from moderated t-test of cLH versus cNLH.

SEQ_ID	Gene Symbol	Transcript	Mean					Standard Deviation					Fold change (cLH v cNLH)	P value
			WT Naive	WT LH	WT NLH	cLH	cNLH	WT Naive	WT LH	WT NLH	cLH	cNLH		
NM_001037140	Pcdhga1	protocadherin gamma subfamily A, 1	259	257	242	91	420	131.55	78.16	167.96	28.577	106.94	4.62 -fold down	0.01
NM_001037139	Pcdhga2	protocadherin gamma subfamily A, 2	3030	2371	3200	3884	2720	698.58	828.19	1051.44	897.501	347.84	1.43 -fold up	1.00
NM_001037154	Pcdhga3	protocadherin gamma subfamily A, 3	1559	1457	1809	1658	1535	388.46	422.63	625.44	593.598	221.60	1.08 -fold up	1.00
NM_001037137	Pcdhga5	protocadherin gamma subfamily A, 5	1220	1203	1260	1209	1270	238.82	263.64	322.53	211.594	184.43	1.05 -fold down	1.00
NM_001014773	Pcdhga7	protocadherin gamma subfamily A, 7	6989	6703	7479	6157	8162	1469.72	1773.03	3101.46	2018.689	1771.63	1.33 -fold down	1.00
NM_001037156	Pcdhga8	protocadherin gamma subfamily A, 8	476	480	511	688	410	106.50	170.74	158.74	133.203	88.47	1.68 -fold up	0.53
NM_001037158	Pcdhga9	protocadherin gamma subfamily A, 9	659	577	803	997	550	100.13	213.66	139.19	284.148	73.71	1.81 -fold up	0.62
NM_001037135	Pcdhga10	protocadherin gamma subfamily A, 10	1506	1400	1677	1362	1482	375.16	393.07	622.59	366.453	236.40	1.09 -fold down	1.00
NM_001012215	Pcdhgb7	protocadherin gamma subfamily B, 7	5000	4711	4108	4597	5931	732.20	445.04	2323.69	990.039	623.53	1.29 -fold down	1.00
NM_001037153	Pcdhga11	protocadherin gamma subfamily A, 11	638	741	924	1261	485	116.51	229.59	288.95	248.271	73.65	2.60 -fold up	0.01
NM_001037159	Pcdhgb8	protocadherin gamma subfamily B, 8	3981	3526	3519	2958	3938	524.43	701.27	1597.59	598.358	609.38	1.33 -fold down	1.00
NM_001037337	Pcdhga12	protocadherin gamma subfamily A, 12	721	655	842	772	895	142.75	112.80	217.49	196.394	138.01	1.16 -fold down	1.00
NM_053943	Pcdhgc3	protocadherin gamma subfamily C, 3	3945	3295	4238	4094	3570	860.93	806.29	774.04	934.867	551.60	1.15 -fold up	1.00

Table 4: List of transcripts to matching those of the γ-protocadherins. Entries are listed according to organization in the genome. Some γ-protocadherins might not be listed.

SEQ_ID	Gene Symbol	Transcript	Mean					Standard Deviation				
			WT Naive	WT LH	WT NLH	cLH	cNLH	WT Naive	WT LH	WT NLH	cLH	cNLH
NM_001004080	Gsn	gelsolin	5212	6614	5541	5721	5021	976.1	1890	597.5	1064	1083
NM_001013086	CapG	capping protein (actin filament), gelsolin-like	179	207	207	112	260	27.29	72.53	42.22	7.548	33.83
NM_198748	Scin	scinderin	30	27	29	34	31	5.310	3.636	2.600	11.32	3.714
NM_021593	Kmo	kynurenine 3-monooxygenase	29	43	35	39	119	10.91	29.91	19.33	7.368	27.84
NM_023973	Ido1	indoleamine 2,3-dioxygenase 1	36	33	37	31	34	10.24	8.575	4.993	5.618	7.692
NM_001013164	Ccbl1; KatI	cysteine conjugate-beta lyase, cytoplasmic	1399	1627	1507	1641	1680	323.7	282.1	160.1	239.2	324.6
NM_017193	Aadat; Kat2	aminoadipate aminotransferase	1694	1839	1526	1860	1377	438.8	396.6	356.2	265.7	343.0
NM_001015037	Kat3	kynurenine aminotransferase III	1900	2139	1592	2368	2171	438.9	410.6	382.5	132.7	421.5
NM_012682	Ucp1	uncoupling protein 1 (mitochondrial, proton carrier)	39	40	48	31	29	12.07	12.18	28.26	9.170	3.778
NM_019354	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	19526	20397	19263	15241	20166	4072	6744	1612	1970	2715
NM_013167	Ucp3	uncoupling protein 3 (mitochondrial, proton carrier)	82	106	82	186	44	57.83	71.23	44.98	48.46	6.950
NM_033230	Akt1	v-akt murine thymoma viral oncogene homolog 1	9292	9548	10058	10037	9931	1610	1307	493.6	1152	1462
NM_017093	Akt2	v-akt murine thymoma viral oncogene homolog 2	5400	5037	5659	5690	4897	672.4	1147	335.4	1143	565.0
NM_031575	Akt3	v-akt murine thymoma viral oncogene homolog 3	15920	11796	13494	14034	14059	5770	4610	5949	3750	3372

Table5: List of transcripts relevant to the genes listed in Table 3 and their expression levels as reported by microarray signal intensity values. Entries are organized based on relavance to particular entries in Table 3. For most entries individual variation is too great to make claim regarding the different treatments.
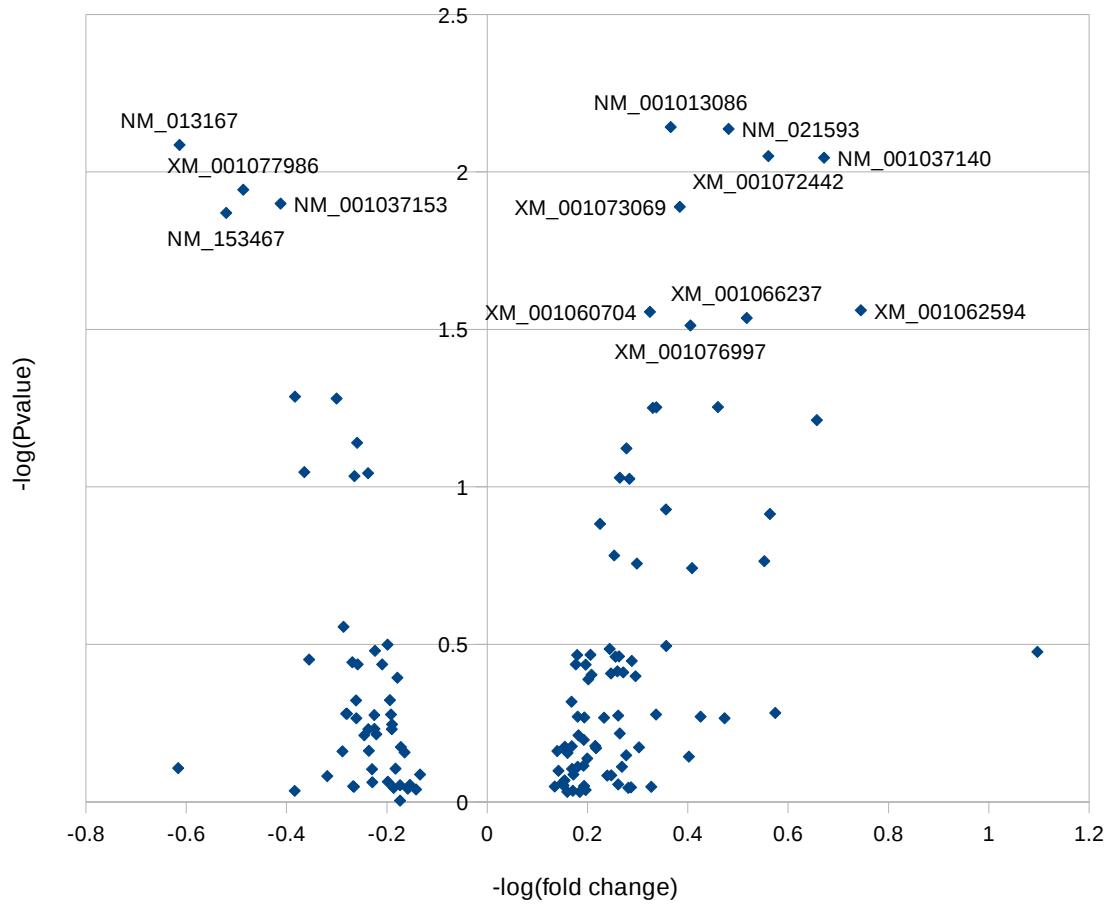
Figure 1: Volcano Plot of Microarray Results. Individual data labels are attached to points depicting transcripts with -log(p) > 1.5, which corresponds to p < 0.05. Transcripts with a -log(p) = 0 were excluded from the graph.

CapG

      CapG is an actin capping protein of the gelsolin family. Besides the obvious roles in regulating the length of actin fibers, it has been shown to be imported into the nucleus and localized within the nucleolus at sites of transcription from rDNA genes (Hubert et al. 2008; De Corte et al. 2004). Additionally it has been localized to the centrisome where it may effect coordination between the actin and microtubule cytoskeletons. Later in mitosis, it is found at the midbody (Hubert et al. 2009). Lastly, a computational paper, seeking to predict protein-protein interactions in the post synaptic density has suggested CapG as potentially being located at synapses (Bar-shira & Chechik 2013).

      Among the gelsolin family, CapG, adseverin, and gelsolin share not just homology but the same domain organization (Arai & Kwiatkowski 1999). During development, these three are expressed in a complementary pattern. Within our array, the most abundant of the three was gelsolin, with low level expression of CapG and virtually no adseverin present (Table 5). It is hard to reconcile a general role in glutamatergic synapses with such low expression. On the other hand, our samples of hippocampal tissue included the entire hippocampal formation, including the dentate gyrus. Therefore the result might be tied to its role in cell division and the presence of neural progenitors in the dentate gyrus.

Kynurenine 3-monooxygenase

      Kynurenine 3-monooxygenase (*Kmo*), also known as kynurenine 3-hydroxylase, catalyzes the addition of a hydroxyl to kynurenine to form 3-hydroxykynurenine. Most non-potein tryptophan within the body is converted to kynurenine by tryptophan 2,3-dioxygenase (TDO) or indolamine 2,3-dioxygenase (IDO) (Knox & Mehler 1950; Schwarcz & Pellicciari 2002). Elevated TDO/IDO activity due to immune activation or stress alters tryptophan metabolism (Capuron et al. 2003; Connor et al. 2008).

Formation of 5-hydroxytryptophan competes with and inhibits formation of kynurenine thus affecting a central component of the serotonin hypothesis. Kynurenine itself can be broken down along two branches. One generates the NMDA antagonist kynurenic acid. The other, the kynurenine-nicotinamide adenine dinucleotide (NAD) pathway, begins with KMO hydroxylating kynurenine into 3-hydroxykynurenine. 3-hydroxykynurenine and the next intermediate, 3-hydroxyanthranilic acid, promote the formation of free radicals. The other products of this pathway are quinolinic acid, picolinic acid, and NAD. Quinolinic and picolinic acids are NMDA agonists and have axiogenic effects. In kynurenine metabolism, the kynurenic acid side is generally neuroprotective (Zunszain et al. 2010) and anxiolytic (Lapin 1998); in contrast, the products of the KMO branch of the pathway are axiogenic (Oxenkrug 2010), neurotoxic (Smith et al. 2009; Okuda et al. 1998), and generally viewed as promoting neurodegeneration (Zunszain et al. 2010).

Normally in the rat brain, kynurenine aminotransferase II (*KatII*), which catalyzes the first step in the kynurenic acid branch, is expressed at much higher levels than Kmo (Connor et al. 2008). Immunological challenge can increase the expression of both *IDO* and *Kmo*; but it is not yet known how significant a physological effect the increased expression would cause. In the rat models the array data suggests a tenfold difference in expression in the cNLH rats and a 48-fold difference in cLH between *KatII* and *Kmo*. This must be considered in light of the fact that the high degree of uncertainty in the measurment of *KatII* expression (Table 5) means that a larger sample size would be needed to determine if these relative differences in expression hold up.

Uncoupling Protein 3

Uncoupling proteins function in the mitochondria to protect against reactive oxygen species (ROS) by allowing proton leak across the mitochondiral inner membrane (Mailloux & Harper 2011). As a consequence, they also effect the efficiency of converting the energy released from the catabolism of pyruvate to ATP (Cioffi et al.

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2009; Azzu et al. 2010; Divakaruni & Brand 2011; Mailloux & Harper 2011). The founding member, *Unc1* is the sole thermogenic uncoupling protein and is expressed in brown adipose tissue in order to allow heat production (Mailloux & Harper 2011; Azzu et al. 2010). *Unc2* and *Unc3* are regulated in a way that is complex and respond to various stimuli including glucose sensing and increased generation of ROS, with *Unc2* widely expressed and *Unc3* expressed primarily in skeletal muscle (Azzu et al. 2010; Mailloux & Harper 2011). Given the much low expression of *Unc3* and the higher expression of *Unc2* (Table 5), *Unc2* is the main uncoupling protein active in the brain. Thus it is unlikely *Unc3* plays any significant role and is unlikely to be worth further analysis.

LOC684913

This transcript has been identified as alpha-fetoprotein related gene (*ARG*) which is a member of the albumin family of genes (Naidu et al. 2010). It is expressed in the perinatal mouse liver at low levels. The primate homolog is predicted to be a pseudogene and while intact in rodents, exhibits low expression and multiple splice variants including some generating frame shifts. It is not known if the gene has much functional significance or if the transcripts generate a protein product. *ARG* was not considered a viable choice for further study.

Cmc1

The COX assembly mitochondrial protein 1 (*Cmc1*) produces two mRNA transcripts. Transcript variant one encodes a 106 amino acid polypeptide, while transcript variant two encodes 66 amino acids and has a distinct 3' end and 3' untranslated region. Originally identified by sequence analysis in *Saccharomyces cerevisiae* as twin CX_9C mitochondrial protein required for full expression of COX and is involved in supplying copper ions to cytochrome c oxidase (Horn et al. 2008). The

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CX$_9$C proteins are conserved among eukaryotes from plants through animals and are generally present associated with the inner mitochondrial membrane. Structurally, the motif results in a helix-turn-helix with the cysteines providing disulfide bonds depending on the redox state (Longen et al. 2009). For most of the CX$_9$C proteins, including *Cmc1*, deletions show reduced cytochrome c oxidase activity. The elevated expression of a *Cmc1* in the congenitally helpless rat agrees with earlier findings of increased cytochrome oxidase c oxidase activity in the hippocampus of adult cLH rats (Shumake et al. 2002). One note of caution however, is that the transcript that was detected in the shorter transcript variant, while it has the twin CX$_9$C motif, it is not known if this is transcript is translated or if the protein has the same function as the longer transcript.

Solute carrier protein family 35 member D3

The solute carriers of family 35 are nucleotide-sugar transporters. Most members of this family are involved in transporting nucleotide-sugars to the endoplasmic and Golgi compartments where they are used in glycosylation of luminal or extracellular peptides (Ishida & Kawakita 2004). This one was first identified in 2007 due to a naturally occurring intracisternal A-particle insertion in this gene. This retrotransposon insertion occurred in a *Rab27a* mutant line and was the source of a dispute between labs as two labs, using the same line but from different breeders were publishing conflicting reports regarding the role of *Rab27a* as a model for disorders such as Hermansky-Pudlak syndrome and Chediak-Higashi syndrome. These disorders exhibit multiple symptoms arising from defects in trafficking of lysosomal related organelles, with results including blood clotting defects, albinism, and impaired immune function. This isolated mutant has the rather unusual phenotype of "empty" platelet dense granules (Chintala et al. 2007). Within platelets, dense granules are secretory vesicles named for their appearance in electron micrographs as the small molecules within them, namely serotonin, calcium, ATP and ADP, result in an electron dense lumen (McNicol & Israels 1999). The paper characterizing the mutant confirmed

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the absence of serotonin in the empty vesicles and noted that while most expression was in bone marrow and platelets, there was also some expression in the brain (Chintala et al. 2007).

Further work has shown that SLC35D3 localizes to the endoplasmic reticulum (ER) and early endosomes (Meng et al. 2012). While SLC35D3 is not normally located in dense granules, failure to express it in the endosomal compartment leads to a failure to develop mature dense granules. This is despite the traditional view that dense granules probably arise from late endosomes or lysosomes and may indicate that, like melanosomes, these lysosome related organelles consist of components derived from both lysosomal and early endosomal compartments.

The most recent work on Slc35d3 has identified it as playing a role in obesity, with a mutant allele affecting trafficking of the dopamine receptor D1 (D1R) to the membrane (Zhang et al. 2014). Along with reduced plasma membrane localization was an increase in D1R retained within the ER (Zhang et al. 2014; Lobo et al. 2006). The expression and effect of the mutation was observed occurring specifically in striatonigral medium spiny neurons that express D1R; neurons expressing the dopamine D2 receptor showed neither expression nor effect from the mutation (Zhang et al. 2014).

During perfusions, we have noticed that our older selectively bred rats have an unusually large amount of fat. Expression levels for the outbred Sprague-Dawley rats from Taconic place expression on par with the cNLH rats and invariant with regard to outcome, implying that this is not a likely cause of the weight gain.

G substrate

G-substrate (*Gsbs*), is a downstream component in the nitric oxide(NO)/cyclic guanosine monophsphate(cGMP)/cGMP-dependent protein kinase (PKG) signaling pathway (Endo et al. 1999; Nakazawa et al. 2005) and is an inhibitor of protein phosphatases 1 and 2A (Endo et al. 2003). In models on Parkensonism induced by

treatment with MG-132 and 6-OHDA, overexpression of G-substrate protects dopaminergic neurons by inhibiting phosphorylating protein phosphatase 2A and thus protecting Akt from dephosphorylation (Chung et al. 2007). Conversely a RNAi knockdown of G-substrate increased sensitivity to 6-OHDA insult and RNAi of Akt reduced the protective effects of G-substrate overexpression.. In amicrine cells within the retina, G-substrate also provides protection from NMDA-induced exitotoxicity by the same mechanism (Nakazawa et al. 2009). In this case, a deletion of G-substrate increased sensitivity of these cells allowing lower doses of NMDA to trigger apoptosis. Furthermore, calpain mediated degradation of G-substrate could be blocked by ALLN resulting in reduced toxicity in wild type but not $Gsbs^{-/-}$ retina.

Protocadherins-γA1 and γA11

Protocadherins were first identified in 1993 and found similar extracellular cadherin (EC) domains to be present within a wide range of organisms including human, rat, mouse, frog, fly, and worm (Sano et al. 1993). Due to additional conserved amino acids in the protocadherin EC domains, which are also in drosophila fat and other non-canonical cadherins, the authors postulated that the EC domains are closer to the primordial EC domain. However, it is important to note that there are no drosophila genes with cytoplasmic domains matching the vertebrate clustered protocadherins, which is not a catenin binding domain that is found in the classical cadherins. The protocadherins are present in vertebrates from mammals to fish and, in mammals, are organized into three clusters of protocadherins labeled α, β, and γ. The α and γ clusters are organized with tandem arrays of large variable exons, each with their own promoter, CpG island, and transciption start site, followed by three short exons which are shared by the members of the cluster (Wu et al. 2001). The gamma cluster is further subdivided into three subfamilies; A, B, and C; however two of the five C-type protocadherins are located in the alpha cluster. While most of the cadherins share more homology with other members of their cluster, the C-type are an exception as they

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exhibit more conservation between C-type protocadherins in the other cluster than with non-C members in their home cluster. This implies a function distinct from the α, γA, and γB protocadherins.

The variable exons of α and γ clusters each encode six extracellular cadherin (EC) domains, a transmembrane helix, and a short cytoplasmic tail of approximately 90 amino acids; while the constant region encodes an approximately 120 amino acid region (Wu & Maniatis 1999).   The 90 amino acid region intracellular segment encoded in the variable exon, the variable cytoplasmic domain (VCD), directs cellular localization of these proteins. While alphas are expressed on the cell surface the γs are found primarily in the endosomal tubules extending from late endosomes and multivesicular bodies and, to a lesser extent, at synapses.

Protocadherins possess multiple similarities with the classical cadherins, although often with important differences. The extracellular domains demonstrate homotypic binding in *trans* much like the classical cadherins, but differ in the fact that they form tetramers in *cis*, and do so in a promiscuous manner with other protocadherins (Schreiner & Weiner 2010). Cell aggregation experiments have shown that the combinatorial homotypic binding results in aggregation exclusively between cells expressing the same or very similar set of protocadherins. This suggests that these clusters of highly related genes produce a greatly expanded set of molecularly distinct surface interactions capable of serving as a means of providing molecular identification of individual cells or cell surfaces which has been compared to a 'barcode'.

Another similarity between classical cadherins and the protocadherins is in how they are both subject to proteolytic processing. Both can have their extracellular domains removed by metalloproteases, such as ADAM10, and subsequent cleavage by γ-secretase resulting in a soluble intracellular C-terminal domain (CTD) (Bouillot et al. 2011; Haas et al. 2005). In the case of γ-protocadherins, the CTD has a bi-partite nuclear localization signal and acts as a transactivator of pcdh-γ expression (Hambsch et al. 2005).

Expression of protocadherins use a combination of biallelic and monoallelic, combinatorial expression patterns to create a diverse expression pattern. The C-type protocadherins are expressed biallelicly with different members expressed at different times during development (Kaneko et al. 2006). The others are expressed in a monoallelic and combinatorial fashion, resulting in an estimated 10^5 combinations of α, γA, and γB protocadherins possible in cerebellar granule cells (Esumi et al. 2005; Kaneko et al. 2006; Kohmura et al. 1998). Later papers, which have focused on other regions, make estimates not on the number of different expression patterns, but on the number of possible protein tetramers possible and include, when members of the β cluster are included, calculate upwards of 3×10^{10} combination possible (Yagi 2012). These studies do so with the known caveat that they are not adjusting for the likelihood that several combinations would be physically indistinguishable. An *in situ* based survey of expression in the hippocampus revealed that all hippocampal cells express some protocadherin, with individual members expressed in partially overlapping subsets of cells and while some members were favored over others in hippocampus, it was qualitatively like the cerebellar granule cells indicating a similar combinatorial expression pattern (Kaneko et al. 2006; Zou et al. 2007; Frank et al. 2005).

Protocadherins play a role in synaptogenesis and cell survival with multiple phenotypes resulting from loss of expression depending on the brain region affected. A transgenic knockout of the entire gamma cluster resulted in neonatal lethality (Wang et al. 2002). Conditional knockouts using Cre-loxP lines have been used to determine the effect in various regions of the central nervous system. The most common result is cell death, as in spinal cord and retina (Wang et al. 2002; Weiner et al. 2005; Lefebvre et al. 2008; Prasad et al. 2008). The cell death in the spinal cord occurs in a non cell autonomous manner, as *Pcdhg* null cells that migrate into an environment where most cells still express γ-protocadherins survive whereas in the converse situation the migrated wild type cells die (Wang et al. 2002; Prasad et al. 2008). Use of a *Pcdh-γ^{del/del}; Bax^{-/-}* rescues apoptosis, yet still exhibits reduced synaptic density in spinal cord and results in neonatal death (Weiner et al. 2005). In retina, the same double-mutant

exhibits no obvious defect in synaptogenesis (Lefebvre et al. 2008); while in cortex, there is a decrease in dendrite arborization of layer V neurons with a resulting decrease in the thickness of layer I without reductions in synaptic density (Garrett et al. 2012). On the other hand, lentiviral mediated expression of Cre to knockout floxed *Pcdh-γ* in subventricular zone progenitor cells results in newly formed olfactory bulb granule cells that do not successfully mature due to both decreased dendrite arborization and a lack of dendritic spines (Ledderose et al. 2013). The cell death phenotypes appear to be largely due to the loss of the three C-type γ-protocadherins, elimination of γC3, γC4, and γC5 variable exons will phenocopy the full knockout while removal of γA1 through γA3 results in no readily observable defect (Chen et al. 2012). Note that in the case of disruption to the alpha cluster, some different phenotypes are observed, such as defects in arborization of serotonergic axon terminals and altered targeting of olfactory sense neuron axons to olfactory bulb glomeruli (Hasegawa et al. 2008; Katori et al. 2009).

Chapter 4: Validation of genes identified in the array

Kmo, *Slc35d3*, and *Gsbs*

Attempts to quantify the changes in Kmo, Slc35d3, and Gsbs by qPCR were unsuccessful. This was largely due to erratic results at lower concentrations of cDNA input. The low expression levels of these genes likely contributed to most of the troubles in this regard. Without measurements at a sufficient number of concentrations, quantification by qPCR was not possible.

Protocadherin-γA11

While many of the other genes expressed at too low a level to be further examined by quantitative RT-PCR; we switched focus to a gene with higher levels of expression. Protocadherin-γA11 (Pcdhga11) showed both a 2.6-fold change in expression and high enough expression to be subjected to validation by quantitative RT-PCR. We therefore focused further efforts on this gene.

Hippocampal tissue from cNLH (n=3) and cLH (n=3) was harvested and used for qRT-PCR. For each sample and primer pair we performed a series of five five-fold dilutions to construct a standard curve and collect reaction efficiencey. Expression of pcdhga11 relative to Gapdh was calculated by the standard curve method. Expression relative to Gapdh in cLH hippocampus ($6.60 \times 10^{-4} \pm 9.39 \times 10^{-5}$) was 3.24-fold higher than in cNLH hippocampus ($2.04 \times 10^{-4} \pm 1.59 \times 10^{-4}$), consistent with the microarray results.

We then sought to determine if the change in mRNA corresponded to an actual

increase in the gene product. To this end westerns were performed on cLH and cNLH hippocampal extract. After normalization to actin signal, the pcdhga11 band for cLH animals was 1.4 times the intensity of the cNLH band.

Localization of Protocadherin-γA11 within the Hippocampus

We used immunofluorescence to visualize the location of PCDHGA11 within the hippocampus. Using GFAP, NeuN, and MAP2 to label astrocytes, neuronal nuclei, and neuronal microtubules, we attempted to find a cell type or structure that would label structures corresponding to the protocadherin. Staining is fairly ubiquitous, with the most noticeable difference occurring in CA1 where a higher proportion of neurons in the stratum pyramidale are labeled. The labeling was more prevalent in sections of cLH rats, as compared to the cNLH rats, with CA1 PCDHGA11 positive cells occurring in clusters and displaying more abundant labeling of the corresponding stretches of stratum radiatum where it recognized long tracts oriented in the same direction as the radial axons. Other regions, including CA3 and dentate gyrus were comparable, however the cLH brains displayed more well defined staining. Unlike previous studies (Garrett & Weiner 2009), this gene did not exhibit staining within astrocytes, yet we did observe several instances in which an astrocyte made extensive contact with a locations containing PCDHGA11. There was also noticeable staining of the endotheilial layer surrounding blood vessels, corresponding to where astrocytes made contact as part of the blood-brain barrier.

Immunofluorescence of primary neuronal cultures

To better visualize cellular localization and in hopes of finding with what structures PCDHGA11 co-localizes, primary cultures were made from rat hippocampus of both cLH and cNLH rats. Initial work showed that the PCDHGA11 appeared to be present mainly in the cell bodies near the nuclear compartments, consistent with prior

and so far unexplained reports of protocadherin γ localizing to endosomal structures via targeting sequences present in the variable cytoplasmic domain, a region of 120 residue segemnt of the cytoplasmic domain which is part of the variable exon of the protocadherin-γ genes. Unexpectedly, PCDHGA11 was found in all cultured neurons, regardless of animal. This may have to do with the fact that the surviving neurons in a primary culture have been undergoing synaptogenesis in the wake of the disruption of the tissue during establishment of the culture. Alternatively, it could be an important clue invalidating several explanations for the correlation seen between *Pcdhga11* expression and learned helplessness. It has been noted that LTP can lead to increased numbers of synapses between the presynaptic cell and post-synaptic cell. One might have been able to claim the endosomal localization was a result of the protocadherins being in the process of being degraded, however the presence of endosomal labeling in all cells in an *in vitro* culture imply that it is not a case of degradation but is the normal location of these proteins.
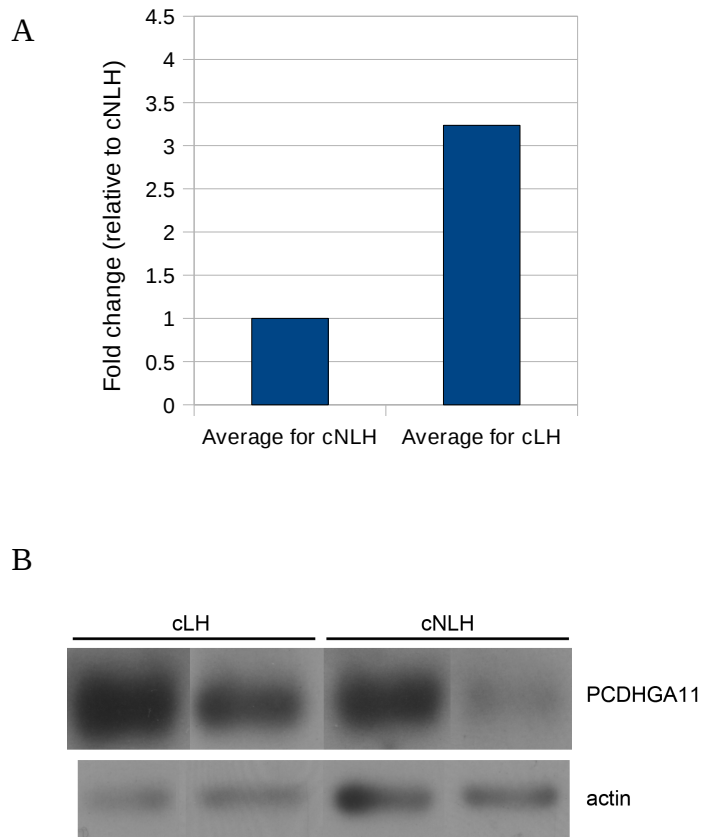
Figure 2: Quantitative PCR and Western Blot Confirming Change in Expression and Total PCDHGA11. (A) qPCR graphed as fold change relative to cNLH expression. (B) Western blot using anti-PCDHGA11 and anti-β-actin as a loading control.
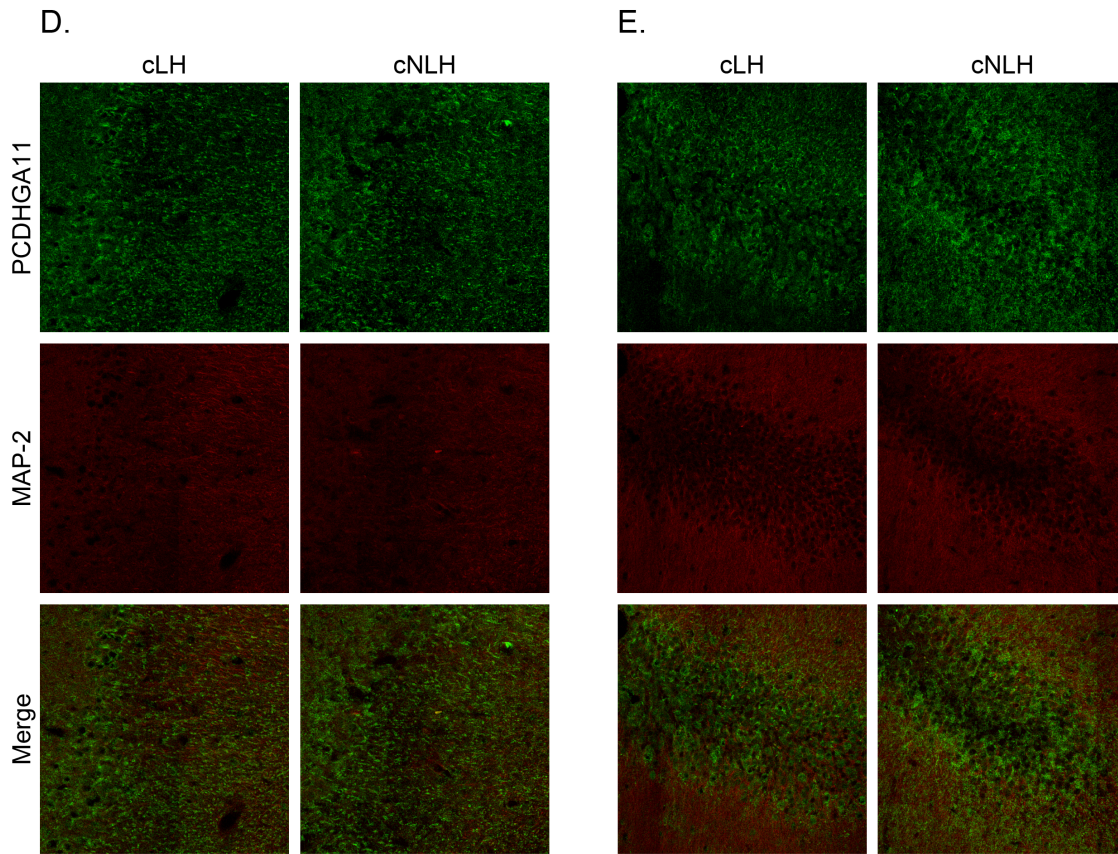
A
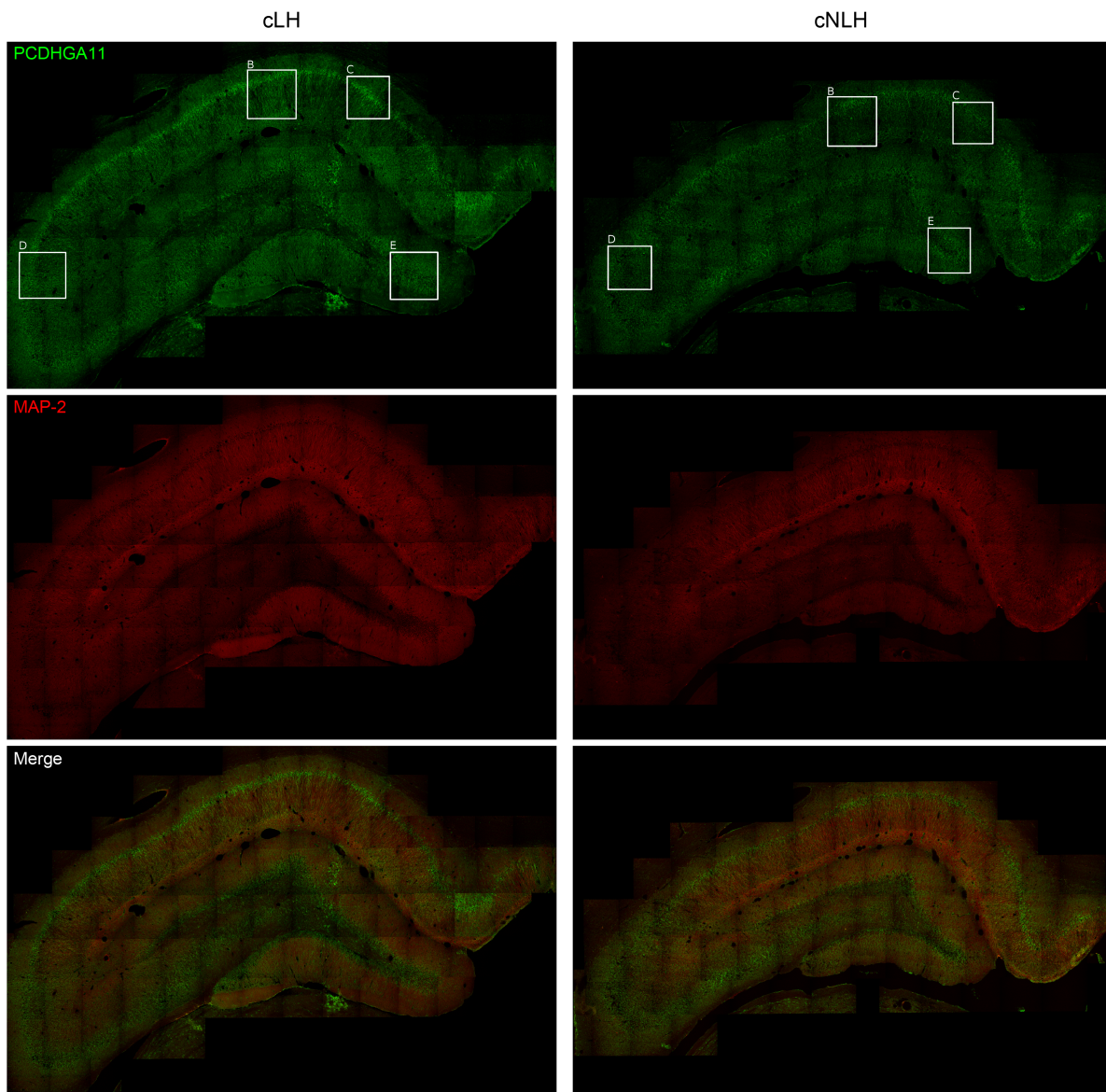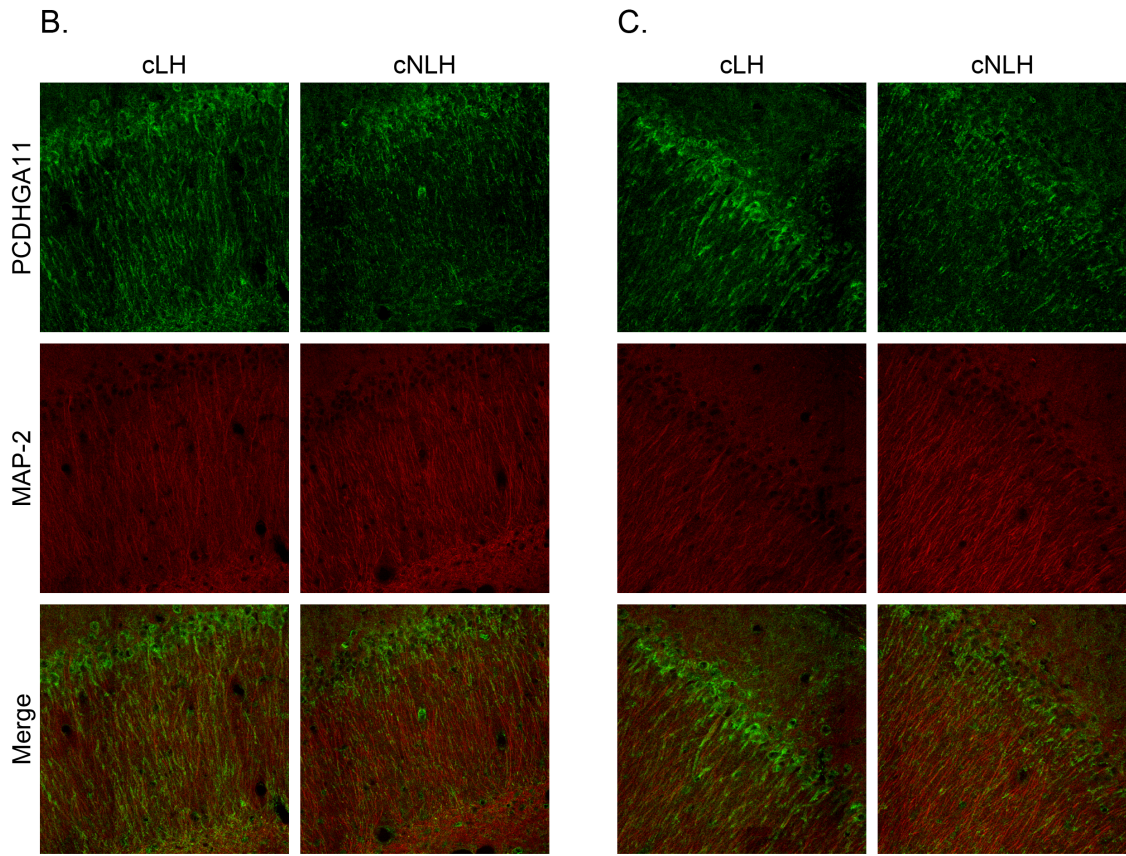
cLH

cNLH

PCDHGA11

MAP-2

Merge

B.

	cLH	cNLH
PCDHGA11		
MAP-2		
Merge		

C.

	cLH	cNLH
PCDHGA11		
MAP-2		
Merge		

Figure 3: Immunofluorescence of Representative Sections of Hippocampus from cLH and cNLH rats. These feature anti-PCDHGA11 in green and anti-MAP-2 in red. (A) Composite images of whole sections constructed from multiple z-projections. White boxes indicate the locations of panels in B-E. (B) CA1 showing stratum pyramidale, stratum radiata, and stratum lacunosum-moleculare. (C) A more medial region of CA1 centered on stratum pyramidale, and showing nearby stratum radiata and stratum oriens. (D) CA3, with the pyramidal layer oriented vertically left of center. (E) The crest of the granule cell layer of the dentate gyrus.

Chapter 5: Discussion

Currently the response rate of antidepressants is low with approximately a quarter of patients not responding to multiple antidepressants. The discovery of medications with more diverse mechanisms may improve this because, until recently, all antidepressant medications acted on targets involved with neurotransmitter metabolism or trafficking. Some researchers have hoped to surmount this by identifying a single pathway or mediator responsible for and common to all depression. Such an ultimate common cause, if it exists, has not been located. An approach far more likely to produce useful benefit is to identify new targets against which drug developers could create medications for treating depression. Already there are some drugs being tested for antidepressant effects via mechanisms distinct from the classical focus on neurotransmitters. These include an HDAC5 inhibitor, sodium butyrate, (Renthal et al. 2007; Tsankova et al. 2006; Schroeder et al. 2007) and the tetracycline antibiotic minocycline (Arakawa et al. 2011). Identifying new potential gene targets for treating depression will open up research into new classes of antidepressant medications and hopefully decrease the frequency of depression patients that do not respond to antidepressants.

Our approach used rats selectively bred for susceptibility to learned helplessness. Since selective breeding based on a behavioral phenotype was blind to possible biological causes it is therefore capable of isolating contributing genotypes that were neither known nor suspected prior to the creation of the selectively bred line. Further, our choice of an array based approach was to avoid any bias of any gene or pathway over another and our choice of an expression array to avoid focusing only on disrupted genes as opposed to changes in expression whether constitutive or in response to the behavioral testing. While the expression pattern present in the LH and NLH wild type rats imply that no gross changes in gene expression need occur in

hippocampus to produce a helpless or non-helpless outcome, the divergent changes in expression in the selectively bred lines imply that alterations in some genes can contribute to the helplessness phenotype.

At first glance, the genes identified as differentially expressed between cLH and cNLH appear to have little to nothing in common, with few involved in the same process or even related to each other. However a closer look at the functions of these genes reveals some interesting theme in the direction of the changes. First *Kmo*, which encodes the protein responsible for the beginning step of the excitotoxic, neurotoxic, anxiogenic branch of kynurenine metabolism is expressed at lower levels in the helpless rat. Similarly, UNC3 works to protect the cell by reducing ROS generation and GSBS exerts a protective effect on the survival factor AKT. Both of these are more expressed in cLH than cNLH. Of course this is all with the caveat that the expression is so low it may be that we can not be sure they are expressed to any significant degree in hippocampus. Two transcripts are expressed at higher levels than the above genes. One of the transcripts expressed at quantifiable levels is the Cmc1 transcript variant 2. This also is upregulated in cLH, but whether this transcript is translated and what the effects of its product are unknown. It is significantly shorter than both the transcript variant 1 and the originally identified *S. cerevisiae* homologue for which the gene's function has been identified (Horn et al. 2008). Thus it is unknown what role, if any, it may play in cytochrome oxidase maturation. The other is protocadherin γA 11, a member of a large family of cadherin related genes.

Unfortunately, the low abundance of these transcripts made further analysis of Kmo, Slc35d3, and Gsbs by qPCR unworkable. A better approach given the situation would have been sectioning and surveying tissue probed with labeled anti-sense RNA to assess where and to what degree the genes in question were expressed and if there is any specific location within the hippocampus where a measurement of expression is most relevant. Moreover, some of these transcripts appear to be part of pathways that one would expect to find more highly expressed in other areas of the brain. For example, SLC35D3 has been reported to effect loading serotonin in secretory lysosome

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related organelles and more recently to be required in striatonigral medium spiny neurons for the dopamine D1 receptor to exit the endoplasmic reticulum (Chintala et al. 2007; Zhang et al. 2014). Therefore one might expect the gene to be most expressed in regions where D1R neurons are present as well as possibly in serotonergic neurons and in regions such as the dorsal raphe nuclei where most serotonergic output originates.

When validating the changes to protocadherin expression we focused on *Pcdhga11*, rather than analyzing both *Pcdhga11* and *Pcdhga1*, as γA11 had higher transcription and therefore a better chance of accurate qPCR results. Additionally, a published effort at mapping expression of all protocadherins in the rat brain demonstrated a complete lack of γA1 expression in the hippocampus as assayed by *in situ* hybridization (Zou et al. 2007). The expression mapping listed several γ-protocadherin genes that were highly expressed in various regions of the brain. Most of those labeled as highly expressed were also listed in the array as having reasonably high expression values including γA3, listed as strong, and γA7, γB7, and γB8 listed as very strong (see Table 4). These genes did not show sufficient fold change between helplessness and non helplessness to be included for further investigated as possibly playing a role in learned helplessness.

On the clustered protocadherins: evidence from gene knockouts

In the fifteen years since protocadherins were discovered, they have come under increasingly intense study, both as the newest form of monoallelic expression and because they may mediate the how neurons within the brain assemble into distinct circuits, especially at a local level. The first few papers focused on the phenotypes of various knockouts, the localization of the protein products and how expression is controlled within these large clusters of very similar genes. Further studies have examined their potential as mediators of intercellular adhesion and subcellular localization. Other works have looked at proteolytic processing or at protein-protein

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interactions. From an early point in the study of these genes, it was proposed that they underlie much of the brain's ability to form specific connections between neurons, a hypothesis that has been supported by several pieces of evidence in subsequent works.

From the studies with knockouts and conditional knockouts we know that γ-protocadherins are required for proper neural development of numerous populations of neurons and may play several roles within the cell. Loss of the entire gamma cluster or the three γC genes results in cell death for many neuronal populations. This can be largely rescued by blocking apoptosis via a *Bax* null mutant. In the *Pcdhg$^{del/del}$ Bax$^{-/-}$* other phenotypes are observed which vary depending on the region studied. The unmasked phenotypes included defects in the development of branching structures such as dendrites as well as reductions in the density of dendritic spines. For instance, interneurons of the dorsal horn of the spinal cord demonstrate apoptosis and reduced density of synapses, although those that do form appear normal (Wang et al. 2002). Both a hypomorphic allele, and the *Pcdhg$^{del/del}$; Bax$^{-/-}$* double mutant showed reduced cell death, but still exhibited the lack of coordinated movement and neonatal lethality seen in the *Pcdhg$^{del/del}$* mutant (Weiner et al. 2005). Cultures of interneurons derived from the hypomorph developed fewer, weaker synapses than control cultures. This cell death is due to apoptosis and is non-cell-autonomous, as indicated using conditional mutants in which *pcdh*-γs are knocked out in select populations of cells, as non-mutant cells migrating into regions where the local population has undergone Cre-mediated excision of the constant exons show reduced survival (Prasad et al. 2008). Even when the cell death phenotype is rescued with a *Bax* mutant, synaptic defects persist (Prasad & Weiner 2011).

The defects seen in spinal cord are not universal throughout the central nervous system. In the retina, loss of γ-protocadherins leads to increased apoptosis in interneurons and retinal ganglion cells, yet the disruptions to synapse formation seen in spinal cord are not present in the retina of the *Pcdh-γ$^{del/del}$; Bax$^{-/-}$* double mutant (Lefebvre et al. 2008). Moreover, the authors reported that the visual system appeared to respond to visual input normally in the double mutant even though a more recent

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paper by the same author revealed defects in dendritic branching of starburst amicrine cells (Lefebvre et al. 2008; Lefebvre et al. 2012). The conditional mutant, when used to create a deletion in cortex, displays a completely different phenotype than seen in either spinal cord or retina. The same $Pcdh\text{-}\gamma^{fcon3/fcon3}$ transgene used to study spinal cord and retina, when combined with *Emx1-Cre* resulted in deletion of the constant region of the Pcdh-γ genes in cortex. In these mice, cortical layer I was reduced in thickness; an effect resulting from reduced arborization of apical dendrites of layer V neurons (Garrett et al. 2012). This occurred without any discernible increase in apoptosis compared to wild type, and the authors claimed no change in synaptic density. Interestingly, the characterization of the mutant was done at later time points than studies examining spinal cord and retina, with the reduction of the dendritic arbors occurring between postnatal day 18 (P18) and P28 (Weiner et al. 2005; Lefebvre et al. 2008; Garrett et al. 2012). Lastly, the olfactory bulb has been a useful structure for studying the effects protocadherins on neurons within the central nervous system. As a region subject to the continual incorporation of new neurons via the rostral migratory stream, the olfactory bulb receives a steady supply of newly developing neurons. Lentiviral-mediated Cre expression was used excise the floxed first constant exon of the γ-protocadherins in subventricular zone neural progenitors. The resulting olfactory granule cells exhibited reduced dendritic arbors much like in cortex as well as a failure to develop dendritic spines.

On the clustered protocadherins: expression and localization

One of the first features noticed during the discovery of the clustered protocadherins was the organization of these genes which reminded many researchers of the organization of the immunoglobulin genes (Ig) and T-cell receptor genes (TCR) (Wu & Maniatis 1999). While the Ig and TCR genes make use of somatic cell recombination to determine the isoform expressed by the immune cell, the *Pcdh* genes do not undergo recombination. Instead expression is determined by monoallelic

expression with each variant's expression independently controlled by its own promoter and CpG island directing transcription from its own transcription start site (Esumi et al. 2005; Wu et al. 2001). Protocadherins are thus expressed in a combinatorial and stochastic manner. Evidence for monoallelic and combinatorial expression was published first for the Pcdh-α gene cluster using single cell RT-PCR to determine which Pcdh-α genes are expressed in each of the sampled cells. This was done in F1 hybrid from two mouse strains with known sequence polymorphisms in the region of interest allowing identification not just of which gene was expressed, but also whether it was expressed from the maternal or paternal allele. Their work found that each cell expressed one to four Pcdh-α genes, and in almost all cases expressed each isoform from either the maternal or paternal allele (Esumi et al. 2005). Following this work members of the same lab examined both Pcdh-α and Pcdh-γ genes in the same cell type, cerebellar Purkinje cells, using an F1 hybrid of a C57BL/6 x JF1 cross instead of the previously used C57BL/6 x MSM mice used previously (Esumi et al. 2005; Kaneko et al. 2006). This work demonstrated the same monoallelic and combinatorial expression in *Pcdh*-γ as in *Pcdh*-α. Furthermore they demonstrated that the isoforms αC1, αC2, γC3, γC4, and γC5, collectively referred to as C-type due to their high homology, were expressed biallelically in almost every cell (Kaneko et al. 2006). Based on the average number of α, γA, and γB expressed per cell, Kaneko R et al estimated that these genes could be expressed in 109,200 different combinations (Kaneko et al. 2006) indicating that the combinatorial expression of these genes could play a role in providing neurons and their cell surfaces with identities allowing for very specific cell-cell adhesion.

The above mentioned expression pattern appears to be present throughout the brain. Unlike other cadherins which are expressed in select populations of cells, the clustered protocadherins are expressed in most if not all subtypes of neurons, yet specific protocadherins will be expressed in some, but not all members of each neuronal subtype (Frank et al. 2005; Esumi et al. 2005; Kaneko et al. 2006; Zou et al. 2007). Thus, the protocadherins are capable of serving as a means of providing a

unique identity to each neuron as the large number of possible combinations of expressed protocadherin leads to a low probability of a neuron expressing the same set of protocadherins as its neighbors.

Although widespread, there are differences in expression temporally as well as spatially. In mice, expression of most pcdh-γ is detectable by embryonic day 10 (E10) and continues through adulthood with a peak between P0 and P9 coinciding with developmentally driven neuronal maturation and synaptogenesis (Frank et al. 2005). While most are slightly downregulated, γC4 is strongly downregulated by P14; in contrast γC5 is upregulated approximately one week after birth and is expressed through adulthood (Frank et al. 2005). In adult rodents expression is most prominent in hippocampus, olfactory bulb, and cerebellum (Zou et al. 2007).

When first discovered, protocadherins were predicted to be involved in cell-cell interaction. Consistent with this, the α-protocadherins were located on the cell surface, primarily at synapses and in unmyelinated axons.   The γ-protocadherins were a different matter, as these genes were localized to both synapses and intracellular organelles with most of the PCDHG in cells are located in the intracellular compartments (Wang et al. 2002; Phillips et al. 2003). A closer look at the behavior of neurons in cell culture revealed that early in neuronal maturation, PCDHG localizes to both axonal and dendritic growth cones (Kallenbach et al. 2003). At later time points, the axonal labeling has ceased, and PCDHG is present only in the soma and in the dendrites. Moreover, the majority of expressed protocadherin-γ is intracellular with approximately 12% of it present in or near synapses in cultured rat neurons (Fernández-Monreal et al. 2010). The remaining protocadherin associates with intracellular organelles, an association that appears to be guided by the variable cytoplasmic domain (VCD) (O'Leary et al. 2011). Within the intracellular organelles, the protocadherins induce the formation of membrane tubules, unusual structures that result from luminal activity of their homophilic extracellular cadherin repeats (Hanson et al. 2010). There is some debate about what function the protocadherins perform. One group has suggested that the localization seen in lysosomes in his experiments allow

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for the formation of "packets" of homogenous groups of protocadherin tetramers which could be delivered to synapses instead of delivering a random set of protocadherins (Hanson et al. 2010). Another competing group demonstrated association of a GFP tagged intracellular protocadherin with a DsRed tagged protein normally retained in the endoplasmic reticulum where both are co-localized inside the dendrites of cultured cells (Fernández-Monreal et al. 2010). The only point on which the groups appear to agree is that the intracellular function of the protocadherins is associated with the cell death phenotype observed in knockouts (Hanson et al. 2010; Fernández-Monreal et al. 2010).

On the clustered protocadherins: properties of the protocadherin protein

As a type I transmembrane protein with extracellular cadherin repeats on the extracellular side, it seemed obvious that these would be proteins mediating cell-cell contact and display surface adhesion properties. Indeed, this is the case for the α-protocadherins which are localized to the cell surface this is the case. The adhesive properties of the γ-protocadherins are, as measured by cell aggregation assays, quite weak. To assess the ability of γ-protocadherins to act in cell-cell interactions, cell adhesion assays were performed by several groups (Obata et al. 1995; Frank et al. 2005; Reiss et al. 2006; Schreiner & Weiner 2010). Replacing the cytoplasmic domain with that of a classical cadherin improved adhesion and altered subcellular localization, redirecting the protein to the cell membrane (Obata et al. 1995). The weak surface adhesion is considered a result of the fact that most of the expressed protocadherin is present in intracellular membranes. The most advanced of the aggregation experiments used cells from a human leukemia cell line (K562) to express tagged forms of various *Pcdh*-γs and combinations of *Pcdh*-γs (Schreiner & Weiner 2010). From their results, it was clear that the specificity of PCDH-γs cell-cell adhesion is not just specific but combinatorial. That is, it is not enough just to have one of several expressed protocadherin in common with another cell for aggregation, but the cells had to express the same combination of pcdh-γs in the same proportions. Others have examined

synaptogenesis specifically in their assessment of the adhesive properties of protocadherins. A set of experiments examining the role of astrocyte-expressed versus neuronal-expressed protocadherins in the formation of synapses in a mixed culture has shown that synaptogenesis requires *Pcdh*-γ expression by neurons and is more efficient when astrocytes also express *Pcdh*-γs (Garrett & Weiner 2009). The adhesive activity is active even when located in intracellular organelles, producing the membrane tubules observed by others. It is not certain what physiological role this plays within the cell. What is known is that the intracellular localization of the PCDHγs is a result of sequences found in the variable cytoplasmic domain and is specific to protocadherins of the gamma cluster (O'Leary et al. 2011). The work detailing this only examined a few of the members of the cluster, thus it is unclear if localization varies by isoform or if all isoforms are present both in intracellular membranes and at synapses or growth cones of developing axons or dendrites.

Outside of synaptogenesis, the homophilic interactions perform another purpose directly related to one of the knock out phenotypes: mediating dendritic self-avoidance (Lefebvre et al. 2012). In order for dendrites to efficiently cover the local area with minimal gaps, dendrites avoid crossing paths. Loss of the pcdh-γ cluster results in dendritic arbors that are disordered, with branches crossing paths, clustering and thus failing to provide even coverage of the surrounding area. This may provide one explanation for the reduced dendritic arbors seen in mutants (Garrett et al. 2012). Rescue with a mutant containing only one functional Pcdhg gene results in restoration of self avoidance but also causes dendrites to avoid those of other cells (Lefebvre et al. 2012). Thus the diversity provides a self-other recognition system for allowing self-avoidance while allowing the dendritic arbors to ignore the dendritic processes of other neurons.

Like the classical cadherins, protocadherins are subject to a similar proteolytic processing by matrix metalloproteases and presenilin-dependant cleavage by γ-secretase. The matrix matalloprotease, identified as ADAM10 cleaves the extracellular domain close to the membrane shedding the extracellular domain and producing the

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membrane-bound C-terminal fragment 1 (CTF1) (Reiss et al. 2006). γ-secretase then cleaves off the cytoplasmic domain as C-terminal fragment 2 (CTF2) (Haas et al. 2005). The γ-protocadherins' CTF2 is subject to proteasomal degradation but can also be translocated into the nucleus where it acts to increase expression of the γ-protocadherins (Hambsch et al. 2005). The ADAM10 cleavage of γ-protocadherins occurs in both a constitutive and inducible manner (Reiss et al. 2006). This inlcudes calcium dependent proteolysis which can be produced by glutamate signaling in neurons but is blocked by the AMPA receptor antagonist CNQX (Reiss et al. 2006). In contrast, the shedding of N-cadherin, which is also often present at synapses, by ADAM10 can be stimulated by NMDA glutamate receptor activity (Reiss et al. 2006). The developmental regulation amounts to a reduction in ADAM10 cleavage of protocadherins as neurons mature and has been linked to neuronal differentiation and interactions between protocadherins (Buchanan et al. 2010). Furthermore, proteolysis can be disrupted by blocking endocytosis with the dynamin inhibitor dynasore (Buchanan et al. 2010).

On the clustered protocadherins: a synthesis

The clustered protocadherins clearly handle several functions within the central nervous system. In some respects there have been promising progress towards an understanding of what and how these transmembrane proteins function. Yet, there are still some functions to the γ-pcdh cluster have largely eluded explanation. The function that has generated the most excitement has been the vast number of distinct surface interactions possible as a result of the combinatorial expression and combinatorial homophilic adhesive properties. Indeed one of the prime reasons for the large number of isoforms may be to create the possibility of a high number of specific interactions. It is thus no surprise that some papers in which an entire cluster has been reduced to a single isoform display relatively minor and in some cases no observable defect or alteration (Hasegawa et al. 2012; Lefebvre et al. 2012). The clustered protocadherins

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are known to form tetramers in *cis* in a promiscuous manner both with other isoforms in the same cluster as well as isoforms in the other clusters. Yet the knockouts of different clusters produce different phenotypes. For instance, the α-protocadherins appear to play a role primarily in myelination (Morishita et al. 2004) and coalescence of axons as well as the branching and penetration of target regions with axon terminals (Hasegawa et al. 2008; Katori et al. 2009). In contrast, γ-protocadherins produce phenotypes associated with the development of dendritic arbors. Presumably however, there would be both Pcdh-α and -γ present at within dendrites, spines, and synapses. In addition to the differing functions based on association with a cluster, there is evidence for subfunctionalization within the gamma cluster itself. Deletion of the three members of the γC family resulted in the same cell death in the spinal cord as observed in knockouts eliminating the entire cluster (Chen et al. 2012). In contrast, loss of γA1, γA2, and γA3 produced no noticable effect. However the deletion of three γC genes removed the entire set of γC protocadherins, while deleting three γA genes still leaves many γA protocadherins intact. If the three subfamilies of the gamma cluster perform slightly different roles, this could explain the difference in effect.

From all these studies, γ-protocadherins appear to play three roles within neurons. They promote cell survival, are required for proper dendritic branching via self avoidance, and take part in the development of synapses (Lefebvre et al. 2012; Chen & Maniatis 2013; Weiner & Jontes 2013; Lefebvre et al. 2008; Garrett & Weiner 2009). The cell death phenotype as well as the intracellular localization are currently the least understood. The dendritic self-avoidance, has been only recently clearly described. The role in synaptogenesis is understood to be involved in later stages of synapse development and does not appear to be required for some regions of the brain even though cultured neurons display and absolute need for *Pcdh*-γ expression to form synapses (Garrett & Weiner 2009; Lefebvre et al. 2008).

Explaining the role in synaptogenesis is probably best done by reference to a paper in which the authors used GFP tagged *Pcdh*-γ*fusg/fusg* to affinity purify and identify protocadherin associated proteins from crude membrane fractions of brain tissue (Han

et al. 2010). The large list of proteins identified include many members of the Pcdh-α and -β clusters as well as over 100 other proteins, mostly PSD associated proteins. A comparison of *Pcdh-γ*^*del/del*^ versus wild type revealed that the size of protein complexes did not significantly change, but the amount of PSD-95, SNAP-25 interacting protein (SNIP), and calcium-calmodulin associated kinase II alpha (CaMKII-α) and CaMKII-γ. Either or both CaMKII and PSD-95 could explain the synaptic deficits seen in the *Pcdh-γ*^*del/del*^ animals. PSD-95 overexpression mimics, and RNAi knockdown blocks, LTP (Kim & Sheng 2004). During LTP, CaMKII is critical to the delivery of AMPA receptor (AMPAR) to the synaptic membrane and also phosphorylates GluR1, increasing the conductance of calcium into the cell through the AMPAR (Lisman et al. 2012). The reported changes in membrane associated PSD-95 and CaMKII in *Pcdh-γ*^*del/del*^ would be expected to produce a shift towards more weak or silent synapses (Kerchner & Nicoll 2008).

The effects on membrane associated PSD-95 and CaMKII combined with the interrelated surface adhesion properties and linked endocytosis and proteolysis of protocadherins implies that in cases where the presynaptic and post-synaptic membrane display the same protocadherins more PSD-95 and CaMKII is present increasing the likelihood that the synapse will undergo activation and LTP. Conversely, in cases where the pre- and post-synaptic cells have very different protocadherin expression patterns, very little PSD-95 and CaMKII would be present resulting in a weak or silent synapse. In the case of astrocytic expression of protocadherin, their presence at the synapse may serve to recruit additional PSD-95 and CaMKII to the synapse compensating for suboptimal matching of protocadherin between the two neurons. This would explain the effects seen in mixed neuron and astrocyte tissue culture experiments (Garrett & Weiner 2009).

Possible role of protocadherin expression changes in the helpless rat

While most of the studies into protocadherins disrupt most or all the genes in a

cluster to determine the function of these genes, our results show a shift in how much a specific protocadherin is expressed. Viewing the altered expression with regard to the role of protocadherins in synapse formation, an overexpression of Pcdhys in the hippocampus may lead to increased random synapses altering the input of the pathway through the fornix to amygdala to the bed nucleus of the stria terminalis (BNST) without changing LTP. This eventually could over activate the LHb which has been shown to drive helpless behavior. This increase in lateral habenular activity is driven by increases in βCAMKII as we have recently shown (Li et al. 2013). If the altered expression may also exert an effect via the self-avoidance that supports the development of dendritic arbors, another effect becomes possible. Within the CA1 region it appears more cells express *Pcdhga11* in cLH than in cNLH. The more homogenous the Pcdh expression among these neurons, the more likely a neuron will encounter another neuron expressing the same set of protocadherins. In such a situation both cells would see the other as 'self' and avoid interaction. This could alter the size or shape of dendritic arbors which could alter patterns of connections without a change in LTP and alter the output of the hippocampus.

Another outstanding question is why this particular protocadherin? Protocadherins demonstrate promiscuous *cis*-tetramerization and specific *trans* association, allowing approximately 60 genes to produce the functional equivalent 10^5 unique cadherins. Yet as far as we know there is no reason why one protocadherin could not be replaced by another in any particular situation. Perhaps we just happened to have animals in which the sets of protocadherins affected happened to all include this one, or perhaps different protocadherins are favored for certain networks. More studies into the expression of the individual members of this family of genes is required.

Whether the changes in protocadherin expression is a result of breeding or the stresses of the learned helplessness paradigm is still an open question. Work assessing the effects of maternal care on the brains of rats have identified epigenetic changes in the three protocadherin gene clusters and resulting changes in expression levels for many of the protocadherins (McGowan et al. 2011). Other work by their group

has identified changes in stress reactivity, expression of various hormone receptors, and hippocampal plasticity (Weaver et al. 2004; Champagne et al. 2006; Szyf et al. 2005). Here we show a change in a particular protocadherin is associated with susceptibility to learned helplessness within a selectively bred line of rats. These changes could relate to either the reduced plasticity seen or in changes in functional connectivity within the brain that occur in association with a depressive phenotype.

The most significant finding was the association of a protocadherin with learned helplessness. Protocadherins are an area of active investigation to determine their roles in astrocyte function, synapse formation, and development of dendrites. Since the publication of the aggregation assays and the initial evidence implying combinatorial expression, it has been regularly proposed that this family plays a role in determining the functional characteristics of the brain. Specifically, they use those words, functional characteristics, which are both vague and imply potentially far reaching effects (Hirayama & Yagi 2006; Yagi 2008). The effects could range from how neurons assemble into networks, providing bias in the ease with which a specific neuron will form strong connections with another neuron, to the types of connections between brain regions, to observed behavioral tendencies. Previous works have shown an altered protocadherin expression in various brain regions in response to a lack of maternal care in rodents, and also in the brains of suicide completers (McGowan et al. 2011; Suderman et al. 2012). Thus, now stress reactivity, suicidal behaviors, and the learned helplessness model of depression all may feature alterations in protocadherin expression.

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