An Initial Attempt to Correlate Prefrontal Cortex mRNA Transcripts with Behavioural Variation in Lewis Rats

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmacology
University of Toronto

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2008

Abstract

Despite virtually identical genomes, inbred animals often vary in phenotype, including behaviour, but the molecular basis of this phenomenon is unknown. Our hypothesis is that differences in behaviour between inbred rats are correlated with differential cortical mRNA transcript levels. 40 Lewis rats were subjected to 5 behavioural tests: two were used to categorize 10 animals into either “high” or “low” phenotype groups. Microarray gene expression profiling was performed for 5 rats from each group. Three main analyses were performed to: (1) identify differential expression between the high and low groups, (2) identify correlations between transcript levels and individual behaviour scores, and (3) determine if the results of this replicate experiment overlapped with a previous pilot experiment. Some array results were confirmed by RT-PCR. We found that this experiment did not replicate the findings from the pilot, however several genes of interest were determined and were validated by RT-PCR.
Acknowledgments

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<th>Full Form</th>
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<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Abl2/Abi-2</td>
<td>Abl-interactor 2</td>
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<td>ASR</td>
<td>Acoustic Startle Reflex</td>
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<tr>
<td>B-actin</td>
<td>Beta actin</td>
</tr>
<tr>
<td>BBS2</td>
<td>Bardet-Biedl syndrome 2 protein</td>
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<tr>
<td>BD</td>
<td>Bipolar Disorder</td>
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<td>BS</td>
<td>Brainstem</td>
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<td>CBL</td>
<td>Cerebellum</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Cort</td>
<td>Cortistatin precursor</td>
</tr>
<tr>
<td>Cox6c1</td>
<td>Cytochrome c oxidase subunit Vlc-1</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine and guanine separated by a phosphate; site of DNA methylation (at the cytosine)</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate putamen</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
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<td>Cryptochrome 2 (photolyase-like)</td>
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<td>Ct/Tc</td>
<td>Threshold cycle</td>
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<td>Dopamine</td>
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<td>Dopamine transporter</td>
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<td>decibel</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>DLPFC</td>
<td>Dorsolateral Prefrontal Cortex</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DSCR111</td>
<td>Down syndrome critical region gene 1-like 1</td>
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<td>EPM</td>
<td>Elevated plus maze</td>
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<td>Fbxl10</td>
<td>F-box and leucine-rich Experiment Two protein</td>
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<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
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<td>Gabrg1</td>
<td>Gamma-aminobutyric acid A receptor, gamma 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC-RMA</td>
<td>Guanine-Cytosine robust multi-array analysis</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>Grin1</td>
<td>Glutamate receptor, ionotropic, N-methyl D-aspartate 1</td>
</tr>
<tr>
<td>GTF2-I</td>
<td>General transcription factor II I</td>
</tr>
<tr>
<td>H3, H4</td>
<td>Histones</td>
</tr>
<tr>
<td>Hbp 1</td>
<td>High mobility group box transcription factor 1</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Hes1</td>
<td>Hairy and enhancer of split 1</td>
</tr>
<tr>
<td>Homer1</td>
<td>Homer neuronal immediate early gene 1</td>
</tr>
<tr>
<td>HPC</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitonial</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>Klf10</td>
<td>Krupple-like factor 10</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial Prefrontal Cortex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>Nfib</td>
<td>Nuclear factor 1B-type</td>
</tr>
<tr>
<td>Nrxn3</td>
<td>Neurexin 3</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PFC</td>
<td>Prefrontal Cortex</td>
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<tr>
<td>Picalm/CALM/</td>
<td>Phosphatidylinositol binding clathrin assembly</td>
</tr>
<tr>
<td>Rims1</td>
<td>protein/Regulating synaptic membrane exocytosis 1</td>
</tr>
<tr>
<td>PPI</td>
<td>Prepulse Inhibition</td>
</tr>
<tr>
<td>PPP1R1A</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 1A</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-Transcription PCR</td>
</tr>
<tr>
<td>SAM</td>
<td>Significance Analysis of Microarrays</td>
</tr>
<tr>
<td>SCZ</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smarca4</td>
<td>SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise</td>
</tr>
<tr>
<td>Snx27</td>
<td>Sorting nexin 27</td>
</tr>
<tr>
<td>Stx3</td>
<td>Syntaxin 3a</td>
</tr>
<tr>
<td>TCAG</td>
<td>The Centre for Applied Genomics</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>Tieg/Klf10</td>
<td>TGFB inducible early growth response (also: Klf10)</td>
</tr>
<tr>
<td>Tmod2</td>
<td>Tropomodulin 2</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
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</table>
1 Introduction

1.1 Statement of problem

Genetically identical, and even near-isogenic, animals have been known to show variability in many physical and behavioural features. The basis of this variation is of basic biological interest, but is also relevant to understanding susceptibility to complex genetic diseases, in which genetic factors clearly influence, but do not fully predict whether a given person gets sick or not. In the context of isogenic organisms, such as inbred laboratory rodent strains or human identical twins, we wished to investigate whether variation in gene transcription and whole cell transcript copy number could potentially account for phenotypic variation. We sought to examine genome-wide expression patterns between isogenic individuals and hypothesized that differences in the transcriptome would correlate with behavioural phenotype in a number of tests relevant to neuropsychiatric disease.

1.2 Hypothesis and research objectives

Inherited gene sequence, epigenetic factors, shared and non-shared environment, and stochastic events can all contribute to phenotype determination, but the interactions among these factors remain an important question in biology and medicine. To investigate these mechanisms affecting phenotype, one can isolate or control one or more of the above factors to observe the effect of other sources of potential variance. This is most practical to implement with genetic factors, because there are both experimental and naturalistic situations where genetic variation is virtually eliminated. In humans, the rare monozygotic (MZ) twin birth presents an opportunity to study differences of non-DNA
origin. For example, MZ twins show discordance for common diseases such as diabetes and hypertension, which are often present in only one twin in spite of a substantial genetic contribution to risk (Wong et al., 2005). In animals, deliberate brother-sister inbreeding over many generations has produced many strains that have high genetic uniformity. Although these highly inbred animals are often superficially similar in appearance, closer examination of individual animals reveals significant phenotypic differences.

Gene expression in normal strains of inbred mice varies between individuals, with differences in the range of 1-3% in liver, kidney or testis (Pritchard, Hsu et al. 2001). Not surprisingly, more macroscopic differences in phenotypic measurements such as body or organ weight show considerable inter-animal variance within inbred strains (Gartner, 1990). To use inbred animal strains to examine the mechanism of non-genetic variation, there is a requirement for minimal genetic variation within the strain. Laboratory strains of inbred mice are derived from a minimum of 20 consecutive brother-sister matings which originate from a single ancestral breeding pair. Statistically, animals within the strain share approximately 98.6% DNA sequence homology for each SNP loci tested (Beck et al., 2000). The oldest inbred mouse strains (DBA, C57BL and BALB/c) have been bred over 150 generations and are essentially genetically identical or isogenic (Beck et al., 2000). Rat inbred strains from different laboratories have been shown to have up to 20% genetic marker heterogeneity in SNP loci between them, this being an important consideration when comparing results between laboratories (Smits et al., 2004).

Genetic factors influence phenotype through heritability ($h^2$), which, in inbred strains, is calculated by comparing the phenotypic variance in an F2 back-cross with the
variance in the parental F1 lines. Heritability estimates for selected behavioural or brain phenotypes in inbred mice range from 0.11 for ethanol tolerance (Rustay and Crabbe, 2004), and 0.35 for dopamine D2 receptor-binding density (Hitzemann et al., 2003) to 0.4-0.6 for locomotor behaviour (Mhyre et al., 2005) and 0.44-0.62 for lifespan (Klebanov et al., 2000). The relatively low heritability values for these traits suggest that non-genetic factors play a substantial role in behavioural phenotypic variance.

We wish to explore the mechanism of non-genetic variation in phenotype and hypothesize that there will be correlations between variation in phenotype and whole-cell mRNA expression levels. The specific objective of this study was to determine whether variation in certain animal behaviours is associated with particular mRNA transcription patterns in the brains of inbred rats.

1.3 Exploratory Approach

To test our hypothesis, we chose to approach this set of questions using the natural variation in behaviour between normal, healthy individuals using a series of behavioural tests performed on a group of inbred Lewis rats. Individuals were tested on four behavioural tests relevant to dopamine system function (Figure 1), and individual animals were rank ordered by their combined scores on these tests. Brains were harvested, PFC removed, and mRNA was extracted for subsequent analysis of genome-wide expression patterns by microarray and qRT-PCR (Figure 2). Microarray analysis of brain mRNA transcription patterns was used to determine differential gene expression patterns that correlated with the specific behaviours observed, and quantitative RT-PCR was used to confirm the mRNA levels of selected candidate genes. The gene list was narrowed by eliminating candidate genes which were annotated as non-coding sequences,
unnamed sequences, predicted sequences, sequences for structural proteins, and by prioritizing transcripts with high relative expression levels that showed correlation with at least one behaviour score (preferably two).

1.4 Gene expression

Expression profiling of the genome or of subsets of the genome has become an important tool in the comprehension of whole system function in combination with genetic and epigenetic approaches. Gene expression profiling is conducted mainly through the use of high-throughput gene expression arrays, and is confirmed by RT-PCR.
Male Lewis rats were acclimatized to the novel housing facilities, then undertook four behavioural tests in sequence.

A) Sugar feeding involved a free-feeding choice between powdered chow and granulated sugar. On the day of testing, a mild stressor was applied (injection of saline, i.p.) as a stimulant.

B) Locomotor tests assessed novelty seeking and anxiety behaviours in the rat, where each rat was allowed to move freely in the box for 60 minutes.

C) The elevated plus maze involves observing the rat for 5 minutes while measuring time spent on the open arm vs. the closed arm.

D) Startle and Prepulse Inhibition of the Acoustic Startle Response: rats were placed in the startle chamber and response to both startle and PPI was measured over 60 pseudorandomized trials consisting of a startle pulse, two different prepulses (15dB apart) and a background pulse.

After these tests were completed, animals were sacrificed and tissues harvested for molecular assessment.
mRNA was extracted from various brain regions. cDNA was generated by reverse transcription. Microarray procedure: cRNA was created from cDNA, and labeled with biotin, then fragmented and hybridized to a GeneChip Expression Array. The array chip was washed, stained, scanned and quantitated, and then the resulting data is processed statistically to determine relative quantity, and background hybridization levels. Intra- and inter-array comparisons were conducted for gene significance testing. Real time RT-PCR procedure: cDNA was created by reverse transcription and then assayed using fluorescent sequence-specific primer assays (Applied Biosystems) in an optical PCR reader. Analysis was conducted to determine the average cycle times, to convert cycle time to relative quantity of RNA to control, and then compared to array data.
1.4.1 Microarrays and the study of gene expression

Microarrays enable the profiling of the entire transcriptome and the detection of large-scale gene expression differences between strains of the same species, tissues, and different treatment conditions, with the potential to identify relationships between expression patterns and genetic, epigenetic or environmental factors (Quackenbush, 2002). Since large quantities of data are generated with each microarray experiment, the analysis is crucial in attempting to determine experimentally significant changes in gene expression (Tusher et al., 2001). Methods include looking for extremes in expression between two individual samples (Eisen et al., 1998), or conducting a network/cluster analysis and attempting to link more subtle gene expression patterns between functionally-related genes (i.e. cancer proto-oncogenes) to discern a mechanism (Eisen et al., 1998; Watkinson et al., 2008). Both analyses can be conducted using prior knowledge of biological interactions, indicated by correlations in expression on the array, which may suggest cooperative effects between genes (Watkinson et al., 2008). Traditional statistical analysis has been modified for the huge number of experimental comparisons conducted in the attempt to reduce the false discovery rate (FDR) of statistically significant gene transcript changes (Tusher et al., 2001). Of primary importance is the rigorous experimental design of the microarray experiment, without which one cannot expect accurate results (Smyth et al., 2003). The measure of gene expression via microarrays has been used successfully to explore transcriptional differences in cancer (Watkinson et al., 2008), development (Semeralul et al., 2006), changes due to physical exercise (Kawai et al., 2007) in response to substance abuse (Singh et al., 2007;
Matsuoka et al., 2008), and behaviour (Hovatta et al., 2005; Nadler et al., 2006; Singh et al., 2007; Cummings et al., 2008).

1.4.2 Oligonucleotide microarrays, the RAE 230 2.0 GeneChip

The Affymetrix RAE 230 2.0 array is a single chip array with 31,042 probe sets of 25-mer oligonucleotide length, each ProbeSet containing 11–20 probe pairs including both perfect match and mismatch probe pairs to control for nonspecific binding. These probe sets represent more than 31,000 transcripts and variants from more than 28,000 substantiated rat genes. The control genes used for this chip are GAPDH, beta-Actin, and hexokinase 1 (Affymetrix array data sheet for RAE 230 2.0 GeneChip). Similarly, the Affymetrix RAE 230A GeneChip is a single chip array with a subset of probes from the RAE 230 2.0 array, with the same oligo probe length (25-mers) and the same number of probe pairs per sequence (11 total). It queries over 15,900 genes (Affymetrix product website, accessed Feb. 20, 2008).

All microarrays, in general, have a similar format, with a variety of probe set oligonucleotide sequences printed onto glass slides for sample hybridization (reverse-transcribed cDNA from sample RNA, genomic DNA for CpG clustering, etc.). GeneChips have multiple replicates per array (internal replicates), which usually give very similar results, however variability between arrays using the same sample (i.e. in experimental replicates) can be a problem depending on variation in the biological sample, array processing, and experimental conditions (Elbez et al., 2006). Affymetrix arrays also include a built-in mechanism for detecting mismatch binding between target transcribed RNA sequences (cDNA labeled with fluorescent dyes) and DNA feature
oligos. This is accomplished by affixing oligos with a single nucleotide mismatch to the target feature region (Affymetrix website, accessed March 20th, 2008). Figure 3 gives an overview of the binding and GeneChip reading process. Since gene transcription to RNA is an essential function in the production of downstream functional protein from DNA code, assaying expression levels across the genome can assist in the detection of phenotypic variability between individuals or groups of individuals at the molecular level. Gene transcription is an important factor affecting the quantity of protein translation (Latchman, 1995).

1.4.2.1 ProbeSets

Multiple ProbeSets for the same gene are occasionally found on the GeneChip expression arrays, and usually have a high degree of correlation in expression levels between them (up to 77% of the time) (Elbez et al., 2006). This can vary depending on whether the ProbeSet is measuring a splice variant of the transcript, or may report transcription of the same RNA with differential accuracy due to degradation of mRNA at different loci, differential efficiency of hybridization to the two ProbeSet, and occasionally because of incorrect annotation resulting in presumptively equivalent probe sets hybridizing different transcripts (Elbez et al., 2006). Multiple ProbeSets can be analyzed with Pearson correlations between hybridization levels to determine whether or not to group these probe sets or to use each ProbeSet separately to analyze the data (Elbez et al., 2006).
Figure 3. Affymetrix MicroArrays.

A) Constructed from 25mers in groups of 11, each DNA strand binds with a complementary strand from the hybridized sample. Mismatch oligos are bound to the chip in addition to exact match oligos to ensure that binding is specific. Each genechip is 1.38 cm square and has 500,000 features per GeneChip array.

B) Only exact matches bind to the oligos. If mismatches bind to the mismatch oligos, nonspecific binding can be calculated during analysis.

C) When laser light is directed on the array, the regions of tagged cDNAs or cRNAs fluoresce, causing entire features to glow. Images from the Affymetrix website (accessed March 28, 2008).
1.4.2.2 Normalization of ProbeSets

Normalization of array data is an important start to analysis and subsequent data-mining. A modified method of normalization (GC-RMA method) models a perfect match of probe set intensity as the sum of Gaussian and exponential distributions for background and signal, respectively, and incorporates mismatches using a model based-on GC content of the oligos (Irizarry et al., 2003). It then uses quantile normalization (Bolstad et al., 2003). Finally, log-scale expression effect and probe effect models are fit robustly to define the expression estimate for each gene ProbeSet.

1.4.2.3 False discovery rate

The false discovery rate (FDR) was proposed by Benjamini and Hochberg (1995) and is defined as the expected proportion of false positive scores among the total number of significant scores. Algorithms based on FDR explore the elimination of false positives and negatives independent of the number of samples being tested, which is important in the analysis of microarray data, since multiple testing is the primary analysis method (Storey JD., 2003). The FDR algorithm is based on P values, and implies the ordering of outcomes instead of applications to multiple hypothesis comparisons (Efron and Tibshirani, 2002). Alternately, Empirical Bayesian methods have been favored for computational analysis of microarrays because of its similarity to the original FDR algorithm. Empirical Bayes inferences examine the probabilities of differentially and non-differentially expressed genes between microarrays. The significance analysis of microarrays (SAM) algorithm, involved in the analysis of FDR, allows for thresholding of expression of genes while correlating them with outcome parameters (i.e. treatment) and can be a powerful method of examining higher or lower expression levels of genes.
across a microarray experiment (Storey J.D., 2003). Additional analysis of microarray experiments is lengthy and intricate, and methodologies are still in development to determine the best statistical analysis for a given array type. This field is growing very quickly and the statistical methods and techniques go beyond the scope of this thesis.

1.4.3 RT-PCR, gene expression, and confirmation of microarray results

An increasingly important method in biological research, RT-PCR is used in accurate and high-throughput expression profiling of selected genes (Vandesompele et al., 2002). The TaqMan® real-time fluorescent PCR system specifically, is ideal when the tissue source for RNA is small in size or is difficult to obtain, as in the case of human clinical samples (see Figure 4). This method requires as few as 1000 molecules of RNA, 200 times less than the quantity required for an RNase protection assay. RT-PCR is quite reliable, with inter- and intra-assay coefficients of variation between measurements being as low as 8% (Proudnikov et al., 2003), and usually involves cDNA amplification of the native extracted RNA transcripts.

PCR reactions have three phases: exponential, linear, and plateau, which refer to the quantity of PCR products still available for the reaction in proportion to the quantity of initial template under ideal conditions (Yuan et al., 2006). Figure 4 demonstrates how the Applied Biosystems TaqMan® assay system provides easy identification of PCR product during real-time data acquisition. RT-PCR can be used for absolute and relative quantification, the latter being a measure of the transcript in question normalized to one or more housekeeping genes. Accurate normalization is a prerequisite for reliable results
Figure 4. Real time RT-PCR and the Applied Biosystems TaqMan Assay System

A) Primers and probe are annealed to the cDNA, and extension begins. When quencher dye and reporter dye are in close proximity, the wavelength of fluorescence is red (seen in e).

B) The probe begins to be displaced by the 5’ nuclease activity of TaqMan Polymerase.

C) Probe is displaced by TaqMan Polymerase and becomes distanced from the quencher dye, fluorescing green.

D) With polymerization complete, fluorescence has shifted towards that of the reporter dye.

E) From Applied Biosystems’ “Real-time PCR vs Traditional PCR” documentation for their 5’ nuclease activity assays, found on the Applied Biosystems’ website accessed March 20th, 2008. Assays for RT-PCR were selected based on availability, amplicon length, coverage of regions of interest (exonic) as well as splice variants.
especially when the biological significance of subtle gene-expression differences is the context for evaluation (Vandesompele et al., 2002).

1.4.3.1 Housekeeping Genes

Common housekeeping genes used in normalization in the rat brain include beta-Actin, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), beta-Actin, cyclophilin and 18S ribosomal RNA (rRNA). These control genes have been selected for their consistency in expression even though there is evidence that stress, aging, drug use, toxins, tumorigenesis and other factors can modify housekeeping gene expression and rRNA (Proudnikov et al., 2003; De Ketelaere et al., 2006). GAPDH and 18S rRNA may be two of the most stable housekeeping genes (Proudnikov et al., 2003) but this depends on the tissue and on the disease state of interest (De Ketelaere et al., 2006).

1.4.3.2 Validation of microarrays

Real-time RT-PCR is a useful tool in the validation of microarrays, since it is the most sensitive and reliably quantitative methods of gene expression analysis. A base-2 logarithmic plot of the fluorescence signal against cycle number will generate a linear graph, from which the log of fluorescent signal can be used to calculate the original quantity of template (Yuan et al., 2006). From this, either absolute or relative quantification of copy number can be ascertained. Absolute quantification uses an internal or external calibration curve to calculate input template copy number. However, relative quantification relies on comparisons between the target gene and a reference gene, such as GAPDH, and the expression of the same gene in a target sample versus reference samples (i.e. a standard curve) (Yuan et al., 2006).
Proper control for real-time PCR experiments requires the generation of a standard curve to account for variation in amplification efficiency between individual primer sets and PCR conditions (Yuan et al., 2006). Amplification efficiency is also dependent on the quantity of native RNA available. Quality control of RT-PCR data can be obtained by examining the correlation between Tc number and cDNA concentration, which may vary between samples and between different reverse transcription reactions used to produce cDNA. A better correlation is between the Tc and the logarithm-transformed concentration of template molecules, the product of which should be a linear relationship (generated by linear regression) for each sample and gene combination (Yuan et al., 2006). However, when Tc > 30, this relationship is no longer linear, and thus represents the lower limit of quantification possible with this method.

1.4.3.3 RT-PCR statistical analysis

The analysis of RT-PCR data for relative cDNA quantification requires the proper selection of control genes. It has been argued that the geometric mean between several housekeeping genes is a more accurate and stable ‘control’ than using a single housekeeping gene, such as GAPDH. Although some (Vandesompele et al., 2002) recommend testing the sample with variety of control genes and then using three housekeeping genes with the least variability (Vandesompele et al., 2002; De Ketelaere et al., 2006) to generate the geometric mean, this is not feasible in small samples with limited quantities of extractable RNA. As a final comment, RNA integrity can drastically affect downstream measurement of cDNA in arrays or in real-time RT-PCR measures. Contamination with protein, genomic DNA, nucleases, and inhibitors of the RT-PCR reaction can all affect RNA quality, and RNA degradation must be controlled during
sample preparation as it can significantly affect quality and subsequent experiments (Fleige and Pfaffl, 2006).

1.5 Epigenetics and the regulation of gene expression

Epigenetic modification of the genome is a method of regulation of complex gene expression patterns, and a cause of variation within a sample population with almost identical genomic DNA (Mund et al., 2006). Epigenetics may be a means of translating environmental influences into transcription at specific genomic sites, which may subsequently affect phenotypic differences (Petronis, 2006)(see Figure 5). The phenomenon of epigenetic differences is acknowledged in isogenic organisms, and applies to nearly isogenic organisms such as inbred animals (Rakyan et al., 2002), resulting in different phenotypic outcomes, and is the result of alterations to the chromatin by CpG island methylation and/or by variable degrees of histone modification (Mund et al., 2006). Methods used to evaluate changes in chromatin, histones and methylation include variants of chromatin immunoprecipitation, CpG microarrays, and bisulphite treatment with subsequent restriction enzyme incubation.

1.5.1 Chromatin alterations

Alterations to the structure of chromatin can influence gene expression – condensed chromatin inactivates gene expression and open chromatin allows for gene expression (Rodenhiser and Mann, 2006). These states of expression are regulated in turn by reversible epigenetic patterns of DNA methylation and covalent histone tail post-translational modifications such as acetylation of the H3 and H4 histones (Petronis, 2006), phosphorylation, methylation, ubiquitination (Turner, 2005; van Vliet et al., 2007),
ribosylation, glycosylation, and sumoylation (van Vliet et al., 2007). Histone tail modifications act to regulate proteins which determine chromatin structure, activating or silencing genes (van Vliet et al., 2007). Non-coding RNAs (both short interfering RNA (siRNA) and microRNA (miRNA)) have also been shown to play a role in plant gene silencing by targeting homologous genes, which they silence by recruiting DNA methyltransferases and histone modifying enzymes (it is still unclear whether this occurs in mammals)(van Vliet et al., 2007).

In mammals, DNA methylation is a post-replication modification predominantly found on cytosine residues of the dinucleotide sequence CpG (Jaenisch and Bird, 2003; Mund et al., 2006). Mammalian DNA has approximately 80% CpG dinucleotides methylated and methylation can inhibit gene expression through the recruitment of transcriptional co-repressors via methyl CpG binding protein recruitment (van Vliet et al., 2007). DNA methyltransferases are an enzyme family comprising enzymes which catalyse DNA methylation at these cytosine residues (Jaenisch and Bird, 2003; Mund et al., 2006). Hypermethylation of a promoter or exonic region of the genome via DNA methyltransferases will silence this region, while demethylation will reinstate expression (van Vliet et al., 2007). Epigenetic regulation of gene expression has been implicated in complex disease such as bipolar disorder, schizophrenia, cancer, autism, lupus, metabolic syndrome, and anxiety and depression (van Vliet et al., 2007).
Chromosomal DNA is covalently bonded to methyl groups (i.e. methylated) at cytosine residues (converting cytosine to 5-methylcytosine) in regions that are not undergoing transcription. Hypermethylation of these regions effectively silences DNA transcription and downstream protein production. Additionally, covalent chemical modifications to histone tails can regulate DNA structure of DNA interacting with the histone, and contribute to RNA transcription. Histone modifications that contribute to changes in expression include acetylation, methylation, ubiquination, sumoylation and phosphorylation. Changes to both methylation patterns and histone modifications can be induced by environmental contributors such as diet, chemical exposure, maternal and social interaction effects, exposure to teratogens in utero, and epigenetic carcinogens.
1.6 Animal behaviour models of human disease symptoms

Animal models that selectively mimic behaviours and states that humans experience are an important tool in psychiatric research, since for obvious ethical reasons human experimentation and tissue sampling is impossible for brain disorders. Many common behavioural tests used to assess schizophrenia and psychosis-related symptoms are affected by dopamine system function, because of the strong effect of this neurotransmitter system in the production and treatment of psychotic symptoms. Since behavioural activity in rats is greater during their dark cycle (Desousa et al., 1998), we performed all of our behavioural assays in the light, where the potential for confounders due to hyperactivity are reduced. Paylor et al. (2006) have shown that the inter-test interval could be as little as 1-2 days without any significant behavioural differences being seen in subsequent tests.

1.6.1 Sugar feeding

The consumption of sweets and other palatable foods has been associated with dopamine and brain opioid release in the limbic system, and can activate opioid receptors in the ventral tegmental area and stimulate dopamine releasing cells in the nucleus accumbens (NAc) (Colantuoni et al., 2001). Rats with increased consumption of sugar have been shown to have increased NAc DA activity in comparison to rats with lower sugar consumption levels (Sills and Vaccarino, 1996), and repeated access to sucrose has been shown to increase DA turnover in the NAc (Hajnal and Norgren, 2002). Minor stressors have been shown to both increase and reduce sugar consumption, dependant on the method of administration. Mild chronic stress has been shown to generate stress-induced anhedonia, which results in the reduction of sugar consumption in rodents.
(Strekalova et al., 2004). However, non-chronic mild stress has been shown to stimulate sugar consumption (Sills and Vaccarino, 1994). Sucrose intake has been shown to correlate with exploratory locomotor activity (reducing it in individuals with lower sucrose intake)(Desousa et al., 1998), self-administration paradigms, elevated plus maze/anxiety and fear-avoidance behaviour and is correlated with acoustic startle reflex response (Desousa et al., 1998). Environmental stressors applied during early development, such as maternal separation, handling, or isolation have been shown to affect anxiety responses in male Sprague Dawley rats (McIntosh et al., 1999; Salzberg et al., 2007). Sugar ingestion has also been postulated to induce anxiety-like behaviour via the formation of free radicals (Souza et al., 2007).

1.6.2 Elevated plus maze

The elevated plus maze (EPM) measures anxiety by determining the amount of exploratory activity during the testing session, and whether or not the animal explores open spaces (i.e. the open arms of the maze) vs. remaining in less threatening locations, such as the closed arms of the maze. Anxious behaviour in rats in this maze is readily relieved with the use of anxiolytic drugs such as benzodiazepines. The results of testing in the EPM have been shown to be predicted by sugar consumption, and possibly by acoustic startle responses (Desousa et al., 1998). Levels of anxiety and EPM performance varies by strain (Shepard and Myers, 2008). Performance in the EPM has associations with increased corticotrophin-releasing factor (Shepard and Myers, 2008), histaminergic neurotransmission in the hippocampus (Alvarez and Alvarez, 2008), and glutamatergic activity in the NAc which incites novelty-seeking in the rat (Alvarez and Ruarte, 2001). Performance has also been shown to be affected by prenatal immune challenge, such as
with lipopolysaccharide (Bakos et al., 2004), which induces inflammation and flu-like symptoms in pregnant dams. Quantitative trait loci for anxiety have been suggested on chromosomes 2, 5, 6, and 7 using the EPM to determine phenotype (Conti et al., 2004).

EPM test reliability is questionable. Results are often contradictory between and within laboratories, or show significant variability for behaviour both in pharmacological experiments and in animals without pharmacological treatment. These differences can be due to simple differences in handling, changes in the housing or testing environment, or variation in lighting conditions (de Araujo Godinho et al., 2007) which may be important for establishing arm preference. Hogg (1996) has written an excellent review of these phenomena and overall EPM reliability as a measure for anxiety.

1.6.3 Locomotion in an Open Field

Locomotion in an open field can be used to as a measure of general locomotor activity, exploratory behaviour, and anxiety, and can be affected by illumination of the testing apparatus as well as other environmental conditions (Tang et al., 2002). Locomotor activity is measured by the distance traveled on the horizontal plane of the open field, where exploratory behaviour is measured as rearing events, measured on the vertical dimension of the apparatus (Tang et al., 2002). This test is thought to cause anxiety because the animal is placed in an unfamiliar situation without any means of escape. Anxiety levels are measured by determining how much time each animal spends in the centre of the apparatus; assuming that the field edges are perceived as “safer” for the animal (Dulawa et al., 1999). The open field test can also measure risk taking behaviour (Marques et al., 2008). Locomotor activity can be affected by androgen levels (Li and Huang, 2006) but has been shown to be unaffected by gender or stress in Fischer
344 rats (Bowman et al., 2006). Locomotor activity can be increased by isolation rearing (Heidbreder et al., 2001; Levine et al., 2007) and psychosocial deprivation, and has been associated with reduced immediate early gene expression levels in the mPFC (Levine et al., 2007; Levine et al., 2008). Psychosocial isolation during rearing can result in a spectrum of symptoms that emerge in adulthood and which can be reversed by administration of atypical antipsychotics clozapine and olanzapine, which generate significant increases in DA outflow in the mPFC (Heidbreder et al., 2001).

1.6.4 Startle and prepulse inhibition of acoustic startle reflex

The startle reflex is a whole body response to intense, sudden stimulus, and is normally attenuated in human and animal subjects when a startle stimulus is preceded by a weaker warning stimulus – the prepulse (Hsieh et al., 2006). Prepulse inhibition (PPI) is a measure of sensorimotor gating (Kumari et al., 2008). The reflex, as well as PPI, are highly conserved evolutionarily across many species and can be measured by eye blink, as in humans, or by whole-body movement, as in rodents. PPI is quantified as the reduction of startle response magnitude with the prepulse, and occurs when the interval between the prepulse and startle stimulus is 30-500ms (Hsieh et al., 2006). The latency of the acoustic startle reflex is 8 msec from tone of onset to beginning of electromyolgraphic responses in the hindleg. This indicates that a small number of synapses are involved in the primary acoustic startle circuit, which is thought to be subcollicular (Davis et al., 1982).

PPI can be affected by background noise, prepulse duration, frequency and interval (Hsieh et al., 2006) and is disrupted by increased noradrenergic activity, higher
blood glycine levels (Hammer et al., 2007), and D1 receptor agonists (Ralph and Caine, 2005; Hammer et al., 2007) such as amphetamine (Swerdlow et al., 2003) and may be increased by treatment with olanzapine (Wynn et al., 2007). Disrupted PPI can be rescued by administration of antipsychotics (Swerdlow et al., 2006), and appears to be largely mediated through dopaminergic systems in the rat (Swerdlow et al., 2004). Differences in PPI due to maternal treatment before weaning (Choy and van den Buuse, 2008) as well as with adolescent corticosterone treatment (Zhang et al., 2005; Choy and van den Buuse, 2008) have also been shown to have an effect on PPI via the dopamine system, as a measure of stress response to the test. This stress response shows some lateralization to the left hemisphere of the mPFC (Zhang et al., 2005).

It is suggested that neurons within the caudal pontine reticular formation may mediate the startle response (Sipes and Geyer, 1997), and that PPI is modulated via limbic and cortico-pallido-striato-thalamic circuitry in the rat (Kumari et al., 2005). This response may be a measure of anxiety-related behaviours (Desousa et al., 1998), and is sensitive to changes in dopamine in these brain regions. There is some effect of sex on prepulse inhibition, where estrogen has been shown to increase PPI in rats (Van den Buuse and Eikelis, 2001), and where males are more responsive to weak prepulses than females (Swerdlow et al., 1993). However these confounders do not contribute significantly to assessment of PPI and acoustic startle reflex in a single subject, and is more relevant in assessing populations of subjects.
1.7 Rodent models of disease

1.7.1 Constructs of human disease in the rat

Animal models can facilitate understanding of human disease, especially in areas where research is limited in humans (van den Buuse et al., 2005), i.e. by the collection of tissues such as brain. As complex diseases often combine a spectrum of symptoms that may be impossible to model in animals, selected symptoms can be mimicked in animals and validated based on construct, face or predictive validity. Two widely used models of psychosis, for instance, include prepulse inhibition as a method of observing sensorimotor gating deficits, and hyperlocomotion induced by amphetamine (Tenn et al., 2003; van den Buuse et al., 2005), both of which are seen in human patients and are referred to as endophenotypes of the complex disease (Criteria are listed in Table 1). Sugar consumption may be useful as a monitor of dopamine function in terms of addiction and reward (Colantuoni et al., 2002) (Spangler et al., 2004; Rada et al., 2005) and potentially, of the negative symptoms of schizophrenia (such as anhedonia) when paired with dissociative anaesthetics producing psychotomimetic symptoms in humans, such as phencyclidine (Turgeon and Hulick, 2007).

1.7.2 Strain genetic differences and putative influence on behaviour

Inbred Lewis rats have been shown to vary from other popular inbred strains (such as Fischer 344 rats) in their novelty- and amphetamine-induced behaviours, as well as having a lower basal dopamine transporter function which is consistent with increased novelty-induced locomotor activation (Allen et al., 2007; Gulley et al., 2007). They have low aggressiveness, and share activity levels in an open field with F344 rats, although are
prone to higher level of anxiety especially in the EPM test (Berton et al., 1997). It has been shown that testing Wistar rats across a variety of tests can separate individuals of the same strain, gender, age and environment repeatedly and with confidence, and especially in respect to aversive experiences (Borta and Schwarting, 2005). A study by (Zamudio et al., 2005) has shown that levels of dopamine receptor (D1-like, D2-like, D3) and dopamine transporter binding vary between different strains, and that it may directly affect behaviours such as locomotion, providing some evidence for the differences in behaviour seen between various strains of rats.

1.8 Dopamine pathways and behaviour

Dopamine is involved in multiple brain functions including cognition, learning, attention, mood, sleep, motor activity, motivation, and reward and plays a role in addiction (Alex and Pehek, 2007; Goto et al., 2007). There are three major DA systems: the mesolimbic system, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens, mediating reward; the nigrostriatal pathway, which begins in the substantia nigra pars compacta and projects to the caudate putamen and dorsal striatum, which mediates motor control; and the mesocortical pathway, originating in the VTA and projecting to the prefrontal cortex, which regulates cognitive processes like working memory and selective attention (Alex and Pehek, 2007; Goto et al., 2007). DA projection pathways are very similar between humans and rats, which suggests potential functional validity in some animal model constructs (Figure 6). DA neurons in the VTA that also receive input from mPFC projections send back afferent projections to the mPFC, and DA neurons receiving mPFC input from interneurons direct input to the NAc, modulating DA efflux in this region (Phillips et al., 2003). These pathways interact with
5-HTergic systems, and 5-HT may play a role in the function of DA neurons by interactions with cell bodies and or neuronal terminals (Alex and Pehek, 2007). It has been shown that the depletion of DA in the DLPFC impairs delayed-response performance as much as removing the DLPFC altogether (Diamond, 2007) and that DA release in the PFC is required for cognitive functions such as working memory (Goto et al., 2007). DA release in the PFC can be decreased by long term stress (>2 wks), resulting in hypofrontality and depression-like characteristics, both of which are considered pathophysiological factors in schizophrenia and associated with the negative symptoms of schizophrenia and depression (Goto et al., 2007).

1.8.1 Dopamine and feeding behaviour

DA has been linked with positive reward behaviours, such as the consumption of palatable foodstuffs (Yamamoto, 2006). There is some differentiation between maintenance and pleasure-induced feeding, with the suggestion that DA signaling in the striatum/CPu is important for body weight maintenance feeding, and signaling in the NAc is important for reward-induced feeding behaviour (Palmiter, 2007). Feeding and feeding-evoked changes in DA release have been seen in the mPFC, where both the presentation and consumption of palatable food has been associated with significant increases in DA release in rats. This may be regulated by DAergic neurons in the PFC that project to the midbrain, which have shown increased firing rates in response to salient food reward (Phillips et al., 2003). Additionally, D2 and some D1 receptor agonists suppress feeding behaviour in rats given access to a highly palatable diet. D1 agonists reduce length of time spent feeding and D2 agonists reduce the rates of feeding although D1 agonists are more likely to regulate food intake by central mechanisms and
D1 receptor stimulation may promote satiation (Terry et al., 1995). As such, DA has been heavily implicated in appetitive motivation instead of the ‘liking’ of a foodstuff (Salamone et al., 2007).

1.8.2 Dopamine and locomotor behaviour

Amphetamine, an agent that increases DA in the synaptic cleft, can produce hyperactivity and hyperlocomotion. Increased DA is seen in attention-deficit/hyperactivity disorder, and has been proposed as a mechanism for the positive symptoms of schizophrenia when DA increases in the striatum are observed. Alternately, decreases in DA levels in the PFC have been implicated in giving rise to both negative symptoms and cognitive deficits commonly seen in schizophrenia (Featherstone et al., 2007). The PFC has multiple connections to the motor and sensory cortices which contribute to the regulation of locomotor behaviour, and from the work of Stuchlik et al. (2007; 2008) there appears to be a strong synergistic link between D2 receptor and α1-adrenoceptors in the modulation of locomotor behaviour in the rat. Increased levels of DA in the NAc and the neostriatum have been shown to increase locomotor behaviour during open field testing after habituation has commenced (de Souza Silva et al., 2008). Lewis rats have been shown to have increased novelty-induced locomotor activity relative to F344 rats due to the differential function of DATs (Gulley et al., 2007). Additionally, novel environments including the inescapable testing arena for locomotor behaviour induce a stress-related response as well as novelty-seeking behaviour, and individuals who are high responders to this stimulus tend to be more sensitive to amphetamine-related activity induction, as well as the acquisition of amphetamine self-
administration behaviour, and tend to exhibit greater basal and novelty-stimulated DA levels in PFC, NAc, and striatum (Zhu et al., 2007).

1.9 The prefrontal cortex

This brain region has both anatomical and functional heterogeneity, especially in the mPFC, making generalizations about electrophysiological, behavioural, and physiological responses to various stimuli difficult (Tzschentke, 2001). The PFC is comprised of glutamatergic, dopaminergic and serotonergic tracts. It shares connectivity with the NAc, the hippocampus, in addition to other regions of the brain with complex feedback systems which contribute to executive function and the modulation of responses to psychostimulant drugs, such as cocaine or d-amphetamine. Dose-dependent DAergic activation of behaviour via the PFC has been indicated in response to these particular DA releasing drugs (Pum et al., 2007). The PFC is intimately involved in feeding behaviour, and may play a role in interfacing the taste and the reward systems in rats, especially in dorsomedial neurons in the PFC, which have been shown to respond to gustatory stimulus. The PFC plays a role in inhibitory control over locomotor activity, impulsivity and the response to novelty (Dalley et al., 2007) and has been implicated in the control of fear in conjugation with the amygdala in the EPM (Albrechet-Souza et al., 2008). Lesion studies have shown sensoriagating deficits that resemble those in human schizophrenia, disrupting PPI of the ASR (Tzschentke, 2001). PFC function also varies significantly with sex in behavioural response to stressors (Dalla et al., 2008). Tzchenkte (2001) further reviews the role of DAergic function in the PFC in behavioural regulation.
Figure 6. Dopaminergic pathways in the human and rat brain.

1.10 Schizophrenia: etiology and endophenotypes

The heterogeneity of schizophrenia in its clinical manifestations and etiology has been a significant obstacle to gene discovery, despite the relatively high heritability estimates (Ross et al., 2006). For this reason, biological markers that can segregate pathological subtypes of schizophrenia would be useful for both reducing heterogeneity in research cohorts and in guiding clinical treatment decisions (Gottesman and Gould, 2003). Defining endophenotypes for schizophrenia represents a major strategy for finding a component or dimension of the illness that may be more closely tied to a molecular or genetic etiology (Gottesman and Gould, 2003).

The term endophenotype refers to a discrete and heritable phenotype based on a biochemical test, microscopic examination, or other laboratory test (Gottesman and Gould, 2003). Endophenotypes can be used as a surrogate for a clinical diagnostic category or a subgroup of patients within that category, i.e. schizophrenia. Endophenotypes should be relatively easy to obtain, show high penetrance in the population, be more reliable than clinical diagnosis, and represent a simpler phenotype for analysis than the overall disorder (Pulver, 2000) (Table 1). Genetic analysis of an endophenotype can specify heritability and may eventually result in gene identification (Adler et al., 1999) which can help to understand disease risk if the locus is linked to the disease or contains a causal gene (Price et al., 2006). Endophenotypes are also known as intermediate phenotypes, and can be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive or neuropsychological, and can be useful for bridging the gap between a particular gene and the disease (Gottesman and Gould, 2003).
Kendler and Gardner (1997) have suggested that endophenotypes should act as a refinement filter for a study sample before genetic analysis is pursued. Endophenotypes may improve on ‘hard’ clinical indicators of disease for investigation in that a multivariate phenotype might “have the greatest likelihood of assisting in the search for schizophrenia related genes.” (Iacono, 1998). They may provide a badly needed aid in meta-analysis by breaking population samples into more readily comparable subcategories (Price et al., 2006) that permit more precise diagnosis and therefore reduce noise inherent in the more broadly-defined umbrella diagnosis.

In the search for psychiatric disease genes, endophenotypes may be more easily measured in animal models than the symptom clusters that define the disease in humans (Turetsky et al., 2007). An even stronger case for endophenotypes can be made when independent endophenotypes correlate (Price et al., 2006), allowing for multivariate phenotype analysis that may reveal genetic or epigenetic susceptibility factors, and improve data mining within that multivariate phenotype (Iacono, 1998). Endophenotypes can allow a reductionistic approach to understanding simpler aspects or components of

<table>
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<th>Table 1  Criteria for endophenotype validation</th>
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<td>The endophenotype should:</td>
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<td>- Be associated with the disorder, that it represents a robust, stable and reproducible impairment in an individual, with high-test reliability &amp; state independence</td>
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<tr>
<td>- Be highly heritable, intra- and interfamilial variance should be attributed to genetic rather than environmental factors</td>
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<td>- Cosegregate with illness within families, but be evident in unaffected members at a higher rate than in the clinical phenotype</td>
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<td>- Have measurements that are rapid and easy, and readily acquired in large number of patients with minimal subject cooperation/effort</td>
</tr>
<tr>
<td>- Reflect a discrete and well-understood neurobiological mechanism informative of the pathophysiology of the disorder and be indicative of the action of a limited number of genes</td>
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Modified from Gottesman & Gould (2003), Turetsky (2007)
the overall disease phenotype that would be impossible to achieve by only studying the clinical syndrome. Multiple endophenotypes may represent different dimensions of schizophrenia that may be expressed together in different combinations to produce the full clinical presentation.
2 Experimental Design

The basic experimental design involves testing a group of inbred rats on a series of behavioural tests, and then performing microarray analysis of transcripts from brain, in an attempt to identify transcripts that are present at levels correlated with performance on the behavioural tests. The two experiments described in this thesis detail an initial pilot study and its follow-up study for verification of the molecular targets determined during the pilot. The pilot study (Experiment One) had smaller numbers of animals than the follow-up (Experiment Two), and therefore had less power to detect transcripts that had expression levels correlated with differences in behaviour among rats of the same inbred strain.

Our hypothesis is that phenotypic variation in certain behaviours is associated with particular patterns of mRNA levels in the PFC of inbred rats. To test this hypothesis, two experiments were performed to see whether the gene list generated in the first study was replicated in the second. Experiment One provided genes which were selected for verification based on the strength of statistical relationship between transcript level and
behavioural test score. Genes selected for RT-PCR verification were also chosen based on biological plausibility of their effect on the observed behaviours. Because the initial study was small in size (n=9 rats sampled by microarray) there were concerns that the power of our study might not be sufficient to detect changes at the mRNA level that would have a strong effect on behaviour, recognizing that there are many intervening levels of the nervous system that also may influence these complex behaviours.

Experiment Two was undertaken to increase power and to determine whether the gene list from Experiment One would be partially replicated. This was intended to address the question of whether there are identifiable transcripts that consistently have a strong correlation with the behaviours of interest. Although we feel that this two-part design is quite stringent, and is likely to greatly reduce false positive results, it also introduced a number of complications in data analysis and may have introduced Type II error into our analyses, possibly eliminating genuine hits from our analysis. In particular, the time between the two experiments meant that analysis methods had evolved, and changes in the equipment used to test behaviour also introduced another source of variation between the two batches of animals. A global analysis of both sets of animals was also performed, with the assumption that correlations between specific transcripts and a given behaviour that emerged despite the differences between experiments, would represent much stronger evidence that those transcripts were indeed important in modulating behaviour.
3 Materials and methods

3.1 Experiment One

3.1.1 Animal behaviour

Twenty Lewis rats were assessed with a battery of four behavioural tests: sugar-feeding, locomotion, plus-maze, and startle. Male Lewis rats weighing 200-250 g (n=20) were obtained from Charles River, Montreal, Canada. The rats were acclimatized to the lab for one week before testing. All procedures were approved by the Department of Zoology animal care committee, and conformed to ethical standards of the University of Toronto and the Canadian Council on Animal Care. Rats were singly housed in clear, Plexiglas cages with food and water available *ad libitum*, except as described during the sugar-feeding test. The lights were on from 0800-2000, temperature controlled at 21°C +/- 1°C. All testing was carried out between 0900-1700h.

The order of the testing was as follows: On the 13th day after arrival, the rats were started on the sugar feeding test, which ran for 5 consecutive days. They were then given 3 days with no testing, and on the fourth day were tested for locomotor activity in the open field for one hour. The rats received one day off, and on the next day were tested in the elevated plus maze. One week after the EPM test, the rats were tested for acoustic startle responses. On the 12th day after the startle test, the rats were sacrificed by decapitation and the brains removed. Details of all of these procedures follow.

3.1.1.1 Sugar feeding

For the sugar-feeding test, rats were presented with a choice of granulated sugar or powdered rat chow for a 1-hour period each day for four consecutive days. During the
feeding time, regular chow and water were removed from the cage. The food was presented in two stainless steel feeding cups (8 cm diameter X 4 cm deep) that were placed in the rat’s home cage (Desousa et al., 1998). The presentation of the sugar was varied from left to right in the cage on a daily basis to prevent the development of a side preference. The cups were weighed before and after presentation to determine the quantity of sugar eaten. Care was taken to measure any spillage. On the fifth day, the rats received a saline injection (1 ml/kg, i.p)(Desousa et al., 1998) and the sugar and powdered chow were presented and measured as usual. The saline injection was used as a mild stressor to stimulate feeding on the test day (Sills and Vaccarino, 1994). Data from this day was used as the sugar intake measure for each rat.

### 3.1.1.2 Locomotion

On the fourth day following the end of the sugar feeding test period, the rats were tested for locomotor behaviour in a novel open field. Rats were placed individually in chambers measuring (34×33×22.5 cm) with wire mesh floor and Plexiglas top and sides. Locomotor activity was measured via two infrared emitters and corresponding detectors positioned 11 cm apart and 3 cm above the grid floor. Boxes were interfaced with a computer via in-house designed software that measured both total beam breaks and crossovers (defined as a consecutive front and back beam break). Locomotor activity was measured for 60 minutes.

### 3.1.1.3 Elevated plus maze

Rats were tested on a standard elevated plus maze consisting of four opposing arms (10 X 50 cm) meeting at 90° angles. Two of the arms (opposing one another) were
open, with no walls, and the other two arms were enclosed by walls measuring 40 cm in height. The arms met at a centre area measuring 10cm x 10cm. The maze was on a stand elevating it 60 cm above the floor. The rats were placed individually in the centre of the maze and behaviour was observed for 5 minutes. Entries into the open and closed arms, as well as time spent in these arms, were recorded. Plus maze testing occurred two days after the locomotion testing.

3.1.1.4 Acoustic startle

The rats were tested for acoustic startle responses in sound-attenuated acoustic startle reflex (ASR) chambers (Med Associates, grid rod cage measuring 7.5” x 3.6” x 4.2”). Background noise was set at 70dB of white noise. Rats received a 5-minute acclimation period followed by 60 presentations of 110 dB stimuli with an interstimulus interval of 55-65 seconds. Each stimulus was presented for a period of 30 msec, at a frequency of 5000Hz. Startle responses were measured and recorded by the software associated with the startle apparatus (Med Associates, Startle Reflex version 4.10). Data for each rat was averaged across all trials to come up with the average startle value. Acoustic startle testing took place one week after the plus maze test.

3.1.2 Animal grouping and tissue extraction

A quantitative score was obtained for all four tests for each rat. The rat behaviour scores were rank-ordered, with individuals at the top and bottom of this ranking classified as belonging to “high” and “low” groups, respectively (Sills and Vaccarino, 1994; Desousa et al., 1998; DeSousa et al., 2000) Rats classified as both high sugar feeders and high locomotors were chosen. The rats were sacrificed by decapitation, and the frontal
cortex dissected: Fr1 and Fr2 anterior to Bregma +4.0 mm without olfactory bulb (Paxinos and Watson, 1986). Brain tissue was immediately flash frozen on dry ice and stored at -80°C until RNA extraction. 2 rats with an intermediate behavioural phenotype were also identified and brain tissue was extracted. The PFC was selected in part because it is both the terminus for dopaminergic neurons in the mesocortical tract, and because it is one of the main brain areas affected by schizophrenia. It also plays a role in inhibiting locomotor and novelty-seeking behaviour (Dalley et al., 2007). This part of the frontal cortex was also chosen because it has simple dissection margins, thus minimizing potential noise resulting from different tissue sample boundaries. Whole brain could have been used, with the drawback that small differences in mRNA levels in only one brain region could be obscured by mRNA from other areas. No localization of separate cortical regions was conducted, mainly because of scarcity of tissue and difficulties in dissecting out such small regions, with the potential for border-contamination resulting from imprecise dissections. Ideally, both approaches could be used, but cost was a limiting factor with microarrays.

3.2 mRNA extraction and microarray hybridization

Approximately 20-35mg of frontal cortex was added to a pre-chilled cuvette containing 600 μl Trizol (Invitrogen, Carlsbad, CA). The tissues were mechanically homogenized and then allowed to settle at 20°C for 2 minutes. The homogenate was then centrifuged at 14,000rpm for 10 minutes at 4°C. Chloroform was added to the supernatant (0.2 ml per 1.0 ml of Trizol), shaken and incubated for 15s at 20°C and centrifuged at 14,000 rpm for 15 minutes at 4°C. RNA was precipitated by adding an equal volume of 70% ethanol (DEPC water) at -20°C and vortexed. The Absolutely RNeasy® RT-PCR
Miniprep kit (Stratagene Inc., La Jolla, CA) was used to purify the RNA (Dolter and Braman, 2001), with a maximum of 700 μl of the organic phase-ethanol mixture per column. DNase treatment consisted of 600 μl of 1x low salt wash buffer added to the column, centrifuged at 14,000 rpm for 1 minute at 20°C. The filtrate was discarded and the column dried at 14,000 rpm for 2 minutes at 20°C. Finally, 55 μl of DNase solution was directly applied to the column, incubated at a 37°C for 15 minutes. 600 μl of high salt wash buffer was then added, then centrifuged at 14,000 rpm for 1 minute at 20°C. The columns were washed twice afterwards with 600 μl and 300 μl of low salt wash buffer respectively. RNA was eluted using 80 μl x 3 of TE (pH= 7.5) at 65°C for 2 minutes and then centrifuged at 14,000 rpm for 1 minute at 20°C, yielding 240 μl of RNA solution.

The RNA was concentrated to 1.2 μg/μl using Microcron YM-10 centrifugal filter device kit (Millipore Corp., Bedford, MA). RNase Erase was obtained from MP Biomedicals (Irvine, CA). Absolutely RNeasy® RT-PCR Miniprep kit was purchased from Stratagene Inc. (La Jolla, CA). Commercial RNase/DNase-free water was acquired from Sigma-Aldrich Inc. (St. Louis, MO). Microcon YM-10 centrifugal filter devices were obtained from Millipore Corp. (Bedford, MA). RNA quality assurance was obtained via the Agilent BioAnalyzer 2100 (Brockville, ON) or NanoDrop ND-8000 spectrophotometer (Wilmington, DE) at the TCAG facilities (Toronto, ON) before preparation for MicroArray hybridization.

Frontal cortex RNA from 9 individual rats was hybridized to microarrays: 3 from the high group, 4 from the low group, and 2 from the intermediate phenotype groups. All microarray procedures were carried out at the Centre for Applied Genomics (TCAG).
facilities at the Hospital for Sick Children (Toronto, ON, Canada). Integrity of RNA was verified by visualization of the 18S and 28S ribosomal RNA bands using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA). 10 µg of total RNA was reverse transcribed, purified and labeled with the ENZO BioArray™ High Yield™ RNA Transcript Labeling kit (Enzo Diagnostics, Inc., Farmingdale, New York), and hybridized to Affymetrix RAE230A rat expression arrays (St. Clara, CA), all according to the manufacturers’ recommended protocols. Array images were generated on the Agilent 2500 scanner using the Affymetrix Microarray Suite software (v5.0).

3.3 Microarray data analysis methods

3.3.1 Data pre-processing

_SNIR Method:_ Array data was loaded into R (v2.1.1) using the Affy package (v1.6.7)(Gautier et al., 2004) of the BioConductor open-source project (Gentleman et al., 2004). Data was investigated for spatial and distributional homogeneity and variance in the spike-in controls, then pre-processed with the RMA algorithm (Irizarry et al., 2003) as implemented in BioConductor (v1.1.3). The data was then written to disk and parsed with Perl scripts into a custom-built Oracle database that employs the MAGE-OM-compliant RAD schema (Brazma et al., 2001). The normalized data were then associated with updated sequence annotation as detailed elsewhere (Boutros et al, in preparation). Briefly, the annotation process involved comprehensive alignment of each target sequence to species-specific EST and NT databases, followed by a poll-based cluster-assignment algorithm that determined the best matching UniGene cluster (Waterston et al., 2002). Following annotation the 15,293 ProbeSet RAE230A array was found to contain about 12,600 distinct UniGene clusters (putative gene-products).
3.3.2 Testing for differential expression

To identify differential expression between the high and low groups of animals we used a standard signal-to-noise ratio analysis. Here, signal was defined as the mean difference in intensities between the two groups, and the noise as the pooled standard-deviation. This type of analysis has been successfully used by many other groups (Golub et al., 1999; Li et al., 2005).

3.3.3 Identification of correlations between behavioural scores and expression profiles

To correlate quantitative measures of behaviour with expression patterns, we also employed an extensive permutation study to identify significant correlations using all 9 (3 high, 2 intermediate, 4 low) animals. We first calculated the Pearson correlation (R) and correlation coefficient ($R^2$) between each ProbeSet and each of the four behavioural scores. Next, to assess the significance of our results we employed a SAM-like algorithm to estimate the null distribution (Tusher et al., 2001a). This involved permutation of the columns (arrays) of the expression matrix, thereby randomizing the association between test-score and expression values. For each randomization, the correlation and correlation coefficient were calculated for each ProbeSet/behavioural-score pairing. Following 25,000 permutations of the expression matrix (representing 6.89% of the total permutation space), the ranking of the actual $R^2$ relative to the estimated null distribution was used as the probability the correlation (and hence $R^2$) was significant for a specific ProbeSet/score pairing. Values were deemed significant at the 0.01 significance level. The R (v2.1.1) function used for permutation-analysis is available on our website.
In addition, to help provide confidence that our analysis has adequately controlled for multiple-testing, we employed a second permutation study. Here, we permuted the original datasets 2000 times for each test-score and evaluated the number of ProbeSets found to be significantly correlated with a behavioural-score at the 0.01 significance level by random chance – this value was found to be 16 in both the positive and negative directions. The number of genes detected as significantly correlated by our analysis (Table 2a) is substantially larger than 16 in each direction for three of the four test-scores, providing additional validation of our analytical method. Finally, to test if the Plus-Maze scores are confounded by the use of pseudo-counts, we verified the correlation cut-offs both with and without pseudo-counts and saw no significant difference in either the ProbeSets selected in the first layer of analysis or in the test-scores score cutoffs determined in the second layer of analysis.

3.3.4 Ontological analysis of gene-lists

To identify functional themes in the set of genes identified as differentially expressed between “high” and “low” groups, or in those found to be correlated with behavioural test-scores, we employed ontological analysis. We used build 148 of the GOMiner software package (Zeeberg et al., 2003).

3.4 Method One

Alternately, Method One was conducted to process array data for further validation based on improved analysis methodologies between the time Experiment One and Experiment Two were conducted.
3.4.1 Statistical analysis of microarrays

The raw data (CEL files) were loaded into the R statistical environment (v2.4.1) and processed with the affy (Gautier et al., 2004) package (v1.12.2) of the BioConductor open-source library (Gentleman et al., 2004). Arrays were tested for spatial and distributional homogeneity, and no arrays were excluded from the analysis. Pre-processing employed the GC-RMA variant of the well-established RMA method (Irizarry et al., 2003), which uses the GC content of ProbeSet oligos to calculate the background on the array. This method was used since it removes (in theory) several sources of bias that are retained in analyses generated by the robust multi-array analysis (RMA) method, and has been suggested to work best for this type of experimental design (Boutros, manuscript in preparation). ProbeSet-wise Spearman correlations between behavioural scores and normalized expression values were then calculated. To assess the significance of these correlations we employed the Best & Edwards method (Best and Roberts, 1975). ProbeSets were annotated with updated gene annotation using UniGene build Rn.161 and Entrez Gene, as downloaded on 2007-02-08.

We correlated quantitative measures of behaviour with expression patterns, having employed an extensive permutation study to identify significant correlations using all 10 animals. We first calculated the Pearson correlation (R) and the correlation coefficient (R^2) between each ProbeSet and each of the four behavioural scores. To assess the significance of our results we employed a SAM-like algorithm to estimate the null distribution. This modified SAM algorithm reduces the FDR when compared to the original SAM algorithm (Tusher et al., 2001b). This involved permutation of the columns (arrays) of the expression matrix, thereby randomizing the association between test-score
and expression values. For each randomization, the correlation and correlation coefficient were calculated for each ProbeSet/behavioural-score pairing. Following 25,000 permutations of the expression matrix (representing 6.89% of the total permutation space), the ranking of the actual $R^2$ relative to the estimated null distribution was used as the probability the correlation (and hence $R^2$) was significant for a specific ProbeSet/score pairing. Values were deemed significant at the 0.01 significance level.

In addition, to assess whether multiple-testing was adequately accounted for, we employed a second permutation study. Here, we permuted the original datasets 2000 times for each test-score and evaluated the number of ProbeSets found to be significantly correlated with a behavioural-score at the 0.01 significance level by random chance – this value was found to be 16 in both the positive and negative directions. The number of genes detected as significantly correlated by our analysis (Table 2b) is substantially larger than 16 in each direction for three of the four test-scores (more than the number expected by chance alone), suggesting that a number of transcripts were significantly correlated with behaviour.

3.5 Real-time PCR (RT-PCR) quantification of selected transcripts

cDNA was synthesized from the same set of RNA used for the microarrays samples using the Omniscript Reverse Transcription kit (Qiagen, Valencia, CA). Expression levels of individual mRNAs were measured with quantitative PCR (ABI PRISM® 7000); using the TaqMan® Assays-on-Demand™ Gene Expression assays (Applied Biosystems Inc., Foster City, CA). A standard curve was generated for each assay using test brain tissue, composed of known serial dilutions, in order to establish the
relationship between threshold cycle ($T_c$) and mRNA concentration. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined for all samples as a control (TaqMan® Rodent GAPDH Control Reagents from ABI), and expression levels of each mRNA were calculated as a ratio of the candidate gene/GAPDH $T_c$ values. Thus, the qRT-PCR assays generate a relative expression level for each mRNA and sample tested, and no absolute quantification was attempted (Freeman et al., 1999).

The abundance of GAPDH was measured to be in the same order of magnitude as that of the candidate genes. All reactions were performed in quadruplicate. Real time RT-PCR p-values were calculated using a two-tailed unpaired t-test with Welch’s correction (unequal variance assumption). Three genes were selected for qRT-PCR quantification: TGFB inducible early growth response ($Tieg$), hairy and enhancer of split 1 ($Hes1$), and Homer neuronal immediate early gene 1 ($Homer1$). The NCBI accession numbers, ABI qRT-PCR assay numbers, and stable gene identifiers of the rat genes quantified are as follows: $Hes1$ (NM_024360; Rn00577566_m1; 29577), and $Homer1$ (NM_031707; Rn00581785_m1; 29546), and $Tieg$ (NM_031135, Rn00579697_m1; 81813).

### 3.6 Experiment Two

Forty Lewis rats were assessed with a battery of five behavioural tests: sugar-feeding, locomotion, plus-maze, startle, and prepulse inhibition of the startle reflex. Male Lewis rats ($n=40$) were obtained from Charles River, Montreal, Canada weighing 200-250 g. The rats were acclimatized to the lab for one week before testing. All procedures were approved by the Department of Zoology animal care committee, and conformed to ethical standards of the University of Toronto and the Canadian Council on Animal Care. Rats were singly housed in clear, Plexiglas cages with food and water available ad
except as described during the sugar-feeding test. The lights were on a 12 hour light: 12 hour dark cycle, with lights on from 0800-2000h. The facility was temperature controlled at 21° C +/- 1 ° C. All testing was carried out between 0900-1700h (Desousa et al., 1998).

The order of the testing was as follows: On the 8th day after arrival, the rats were started on the sugar feeding test, which ran for 5 consecutive days. They were then given 2 days with no testing, and on the third day were tested for locomotor activity in the open field for one hour. The rats received one day off, and on the next two days were tested in the elevated plus maze (EPM), one half of the rats per day. Directly after the EPM test, the rats were tested for acoustic startle responses and prepulse inhibition of the ASR simultaneously over two days. Testing order was decided upon by level of perceived stressfulness to animals, sugar feeding being the most benign, and progressing to more stressful evaluations with the conclusion of the startle test. On the 6th day after the startle/PPI test, the rats were sacrificed by decapitation and the brains removed, dissected, and snap frozen on dry ice. Details of all of these procedures follow.

3.6.1 Behaviour

3.6.1.1 Sugar feeding

The sugar-feeding during Experiment Two was exactly as in Experiment One. Please refer to section 4.1.1.

3.6.1.2 Locomotion

Rats were tested for locomotor behaviour in a novel open field. Rats were placed individually in chambers measuring (20×20×22.5 in) with a solid floor, and left in a
minimal-light room for 60 minutes during which locomotor activity was measured.
Activity was recorded by video camera using value contrast to determine rat from
locomotor box, and distance traveled was calculated by EthoVision behavioural software
(Noldus, Leesburg VA, USA).

3.6.1.3 Elevated plus maze

Rats were tested on a standard elevated plus maze consisting of four opposing
arms (10 X 50 cm) meeting at 90° angles. Two of the arms (opposing one another) were
open, with no walls, and the other two arms were enclosed by walls measuring 40 cm in
height. The arms met at a centre area measuring 10cm x 10cm. The maze was on a stand
elevating it 60 cm above the floor (Desousa et al., 1998). The rats were placed
individually in the centre of the maze and the room darkened, and behaviour was
observed for 5 minutes by video recording, and the subsequent determination of entries
into open arm areas was generated using EthoVision behavioural software (Noldus,
Leesburg VA, USA). Plus maze scores were discarded in the final analysis due to a lack
of reliability of the test (animals regularly fell off of the EPM, possibly due to reduced
and inadequately determined lighting conditions).

3.6.1.4 Acoustic startle and prepulse inhibition of the ASR

Rats were placed in sound-attenuated acoustic startle reflex (ASR) chambers
(Med Associates, grid rod cage measuring 7.5” x 3.6” x 4.2”). Background noise was set
at 70dB of white noise provided by the chamber fans. Rats received a 5-minute
acclimation period followed by 60 presentations of pseudo-randomly rotating trials of:
startle stimulus of 115dB (Tenn et al., 2005) white noise for 40ms duration, prepulse of
82dB white noise for 20ms duration, at 100ms prior to the onset of startle (Tenn et al.,
2005), prepulse followed by startle stimulus, and no noise (i.e. 70dB background). The
average interval between trials was 25 seconds. Startle responses were measured and
recorded by the software associated with the startle apparatus (Med Associates, Startle
Reflex version 4.10). Data for each rat were averaged across all trials to come up with
the average startle value and values for each other parameter, the baseline was subtracted
(average reaction to background 70dB noise level), and %PPI was calculated as shown in
Equation 1 below (Dulawa and Geyer, 2000):

\[
\text{Equation 1:}
\]

\[
\%\text{PPI} = \frac{\text{startle to prepulse} \& \text{pulse}}{\text{startle to pulse alone}} \times 100
\]

### 3.6.2 Animal grouping and tissue extraction

A quantitative score was obtained for all five tests for each rat. The rat behaviour
scores were ranked, then cross-correlated using the Pearson correlation (r). The best
correlations were found between dopaminergic-pathway tests, such as sugar feeding and
locomotion, and as such these two traits were used to rank rats as either ‘high’ or ‘low’,
or as a third category ‘intermediate’ split across a median score (Sills and Vaccarino,
1994; Desousa et al., 1998; DeSousa et al., 2000) selecting for individuals towards the
two extremes of responses rather than at the median. Rats classified as both high sugar
feeders and high locomotors, or low sugar feeders and low locomotors were chosen.
Details about sacrifice and tissue harvesting are stated in section 4.1.5.
3.7 mRNA extraction and microarray hybridization

mRNA extraction was conducted using the same protocol as the Experiment One study, see section 4.2. for further reference. Frontal cortex RNA from 10 individual rats was hybridized to 10 respective microarrays: 5 individuals were selected as being ‘high’ responders as a matter of correlation along dopaminergic pathway tests (sugar feeding, locomotion), and 5 individual rats were selected as being ‘low’ responders along the same scale. All microarray procedures were carried out at the Centre for Applied Genomics (TCAG) facilities at MaRS (Toronto, ON, Canada). Integrity of RNA was verified by visualization of the 18S and 28S ribosomal RNA bands using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA). 10 μg of total RNA was reverse transcribed, purified and labeled with the ENZO BioArray™ High Yield™ RNA Transcript Labeling kit (Enzo Diagnostics, Inc., Farmingdale, New York), and hybridized to Affymetrix GeneChip RAE230 2.0 rat expression arrays (St. Clara, CA), all according to the manufacturers’ recommended protocols. Array images were generated on the Agilent 2500 scanner using the Affymetrix Microarray Suite software (v5.0).

3.8 Statistical analysis of microarrays

Refer to section 3.4.1

3.9 Method Two

Alternately, a second statistical method was undertaken to better compare both Experiment One and Experiment Two array sets, to increase stringency and to reduce noise and potential false positives, and to select genes derived from array data for both Experiment One and Experiment Two for validation, using updated methodology. Each
RNA sample was hybridized to a separate RAE230-2 Affymetrix GeneChip by the TCAG facility (Toronto, Ontario). The raw data (CEL files) were loaded into the R statistical environment (v2.6.2) and processed with the affy (Gautier et al., 2004) package (v1.16.0) of the BioConductor open-source library (Gentleman et al., 2004). Arrays were tested for spatial and distributional homogeneity, and no arrays were excluded from the analysis. Pre-processing employed the GC-RMA variant of the well-established RMA method (Irizarry et al., 2003) using the gcrma package of BioConductor (v2.10.0). ProbeSet-wise Spearman correlations between behavioural scores and normalized expression values were then calculated. To assess the significance of these correlations we employed the Best & Edwards method (Best and Roberts, 1975). Additionally linear models, including intercept terms, were fit to each ProbeSet for each behavioural score. These linear-modeling results were subjected to an empirical Bayes moderation of standard error using the limma package for BioConductor (v2.12.0), which allows simultaneous analysis and comparison between many RNA targets to determine differential gene expression (Wettenhall and Smyth, 2004) as well as a false-discovery rate adjustment for multiple testing (Smyth et al., 2003). The moderated p-value generated through FDR control sets a threshold for an expected proportion of false positives in the selected gene list (i.e. a p-value threshold of 0.05 should result in less than 5% of the genes on the selected list being false positives). For each ProbeSet we calculated the maximum possible fold-change (in log2-space) observed based on the linear modeling coefficients and the range of behavioural scores observed. ProbeSets were annotated with NetAffx annotation (version na25). See Table 2c.
3.9.1 Overlap between gene-lists from Experiment One and Experiment Two

The gene-lists generated by correlative analysis were compared and only 12 ProbeSets were found to overlap, while 630 were unique to one of the two lists. The P-value for such a small overlap was determined using the hypergeometric distribution (R v2.1.0) and found to be 0.98 (i.e. 2% probability of such an overlap occurring by chance alone). Similarly, the gene-lists generated by correlative analysis for each of the four behavioural tests were compared. Not a single ProbeSet was found to be in common between any pair of the four lists. Because the hypergeometric distribution is unable to estimate probability accurately for non-occurring events such as the inability to estimate success for ProbeSets which were eliminated by either non-expression of the gene transcript or non-inclusion on the array, we employed a simple jackknife-like simulation, which is used to resample the data leaving out one sample i.e. calculating probability on N-1 samples, N times (in this case one million) and comparing the probability calculated to that calculated on all N samples. Using a Perl script (available online), we randomly selected, with replacement, 367 elements from a 15,293 element array. We determined the ProbeSet with the maximum number of hits, and retained this score. The entire analysis was iterated one million times, and it was found that the no overlap occurred only 12.8% of the time by random chance, strongly suggesting, although not proving, statistical significance for this result.

3.9.2 Ontological analysis of gene-lists

To identify functional themes in the set of genes identified as differentially expressed between “high” and “low” groups, or in those found to be correlated with
behavioural test-scores, we employed an associative analysis of biological significance of each transcript with its ProbeSet, and uses the biological categorization to sort the ProbeSets for statistical significance. This is otherwise known as an ontological analysis.

We used build 148 of the GOMiner software package (Zeeberg et al., 2003), which uses all genes in the genome as a reference to determine statistical significance for each GO term seen in the results of a microarray experiment. This method has limitations, since genes from the assayed sample that do not result in a highly expressed value on the microarray can never be selected as differentially regulated, and genes that are not printed on the array platform cannot be included in the pool available to significance testing.

### 3.10 Real time RT-PCR confirmation and quantification of selected gene transcripts

Refer to section 3.5 for the protocol used for this Experiment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Semeralul et al., 2006) and B-Actin mRNA levels were determined for all samples as a control (TaqMan® Rodent GAPDH, Beta Actin Control Reagents from ABI), and expression levels of each mRNA were calculated as a ratio of the candidate gene/geometric mean of control Tc values (Vandesompele et al., 2002). Thus, the qRT-PCR assays generate a relative expression level for each mRNA and sample tested, and no absolute quantification was attempted (Freeman et al., 1999).

15 genes were selected for RT-PCR quantification based on a potential gene product functional relationship to the behaviours measured, and selected to be run in an independent sample (i.e. the Experiment Two Experiment). Initially, the original three genes of assessment (Kruppel-like factor 10, or (Tieg) (NM_031135, Rn00579697_m1), hairy and enhancer of split 1 (Drosophila) (Hes1) (NM_024360; Rn00577566_m1),
homer homolog 1 (Drosophila) (*Homer1*) (NM_031707; Rn00581785_m1) were
selected, but with disappointing correlations, other genes were selected for follow up
from the original genelist.

The remainder of the 15 genes selected from the original array data included abl-
interactor 2 (*Abl2*) (NM_005158; Rn00687645_m1), cytochrome c oxidase subunit Vlc-1
(*Cox6c1*) (NM_173303.1; Rn00597770_s1), cryptochrome 2 (photolyase-like)
(*Cry2*) (NM_133405.1; Rn00591457_m1), Down syndrome critical region gene 1-like 1
(*Dscr1l1*) (NM_175578.3; Rn00598058_m1), glutamate receptor, ionotrophic, N-methyl
D-aspartate 1 (*Grin1*) (NM_017010.1; Rn00433800_m1), general transcription factor II I
(*Gtf2i*) (NM_001001512.2; Rn01499727_m1), high mobility group box transcription
factor 1 (*Hbp1*) (NM_013221.1; Rn00566331_m1), neurexin 3 (*Nrxn3*) (NM_053817.1;
Rn00587546_m1), syntaxin 3a (*Stx3*) (NM_031124.1; Rn00579637_m1), tropomodulin 2
(*Tmod2*) (NM_031613.1; Rn00581100_m1), phosphatidylinositol binding clathrin
assembly protein (*Picalm*) (NM_053554.1; Rn00585595_m1), and regulating synaptic
membrane exocytosis 1 (*Rims1*) (NM_052829.1; Rn00583862_m1).

The following genes were selected from the analysis of the second set of arrays:
F-box and leucine-rich Experiment Two protein (*Fbxl10*) (NM_001009504;
Rn01500158_m1), Nuclear factor 1B-type (*Nfib*) (XM_001064131, XM_342854;
Rn01764851_m1), SWI/SNF-related matrix-associated actin-dependent regulator of
chromatin subfamily A member 4 (*Smarca4*) (NM_001107864; Rn01448385_m1),
Sorting nexin 27 (*Snx27*) (NM_152847.1; Rn00596111_m1), and Bardet-Biedl syndrome
2 protein (*Bbs2*) (NM_053618.1; Rn00586096_m1).
The second analytical method was used to select gene transcripts differentially expressed in both experiments: Protein phosphatase 1, regulatory (inhibitor) subunit 1A (\textit{PPP1R1A}) (NM\_006741; Rn00575374\_m1), Cortistatin precursor (\textit{Cort}) (NM\_001302; Rn00563272\_m1), gamma-aminobutyric acid A receptor, gamma 1 (\textit{Gabrg1})(NM\_173536; Rn00589841\_m1), and cyclin-dependent kinase 5 (\textit{Cdk5})(NM\_004935; Rn00590045\_m1).

3.11 General chemicals and reagents, other laboratory supplies

The following reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO): diethylpyrocarbonate (DEPC), ethidium bromide, sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl). Ethanol was obtained from Commercial Alcohols Inc. (Toronto, ON). Trizol was purchased from Invitrogen Life Technologies Corp. (Carlsbad, CA). Chloroform was obtained from VWR International (Mississauga, ON). Non RNAse-free water used in solution preparation was purified by Milli-Q UFplus system with resistance of 18.2MΩ\cdot cm (Millipore Corp., Bedford, MA). Disposable plasticwares including microcentrifuge tubes, PCR tubes and pipette tips, were purchased from DiaMed Lab Supplies Inc. (Mississauga, ON) and Axygen Scientific Inc. (Union City, CA). RNAse Erase was obtained from MP Biomedicals (Irvine, CA). Absolutely RNeasy® RT-PCR Miniprep kit was purchased from Stratagene Inc. (La Jolla, CA). Commercial RNase/DNase-free water was acquired from Sigma-Aldrich Inc. (St. Louis, MO). Microcon YM-10 centrifugal filter devices were obtained from Millipore Corp. (Bedford, MA). RNA quality assurance was obtained via the Agilent BioAnalyzer 2100 (Brockville, ON) or NanoDrop ND-8000 spectrophotometer.
(Wilmington, DE) at the TCAG facilities (Toronto, ON) before preparation for MicroArray hybridization.

Omniscript Reverse Transcription kit (Qiagen Corp., Valencia, CA) and OligodT primers (Cortec DNA Services, Kingston, ON. Cat. 9275-05, sequence TTTTTTTTTTTTTTTTTTTT, OD =0.5). The TaqMan Universal PCR Master Mix (with AmpErase UNG) and TaqMan® Assays-on-Demand™ Gene Expression Assays were purchased from Applied Biosystems Inc. (Foster City, CA).
4 Results

We sought to determine whether brain transcription patterns in rats is correlated with behaviour among nearly genetically-identical individuals from an inbred strain. We found that there were transcript levels at the array level which were different between high and low scoring rats for each of the behavioural tests. However, there was little overlap in the specific transcripts between behaviour tests, nor between different batches of animals tested under slightly different conditions.

4.1 Behavioural test analysis

Experiment One was conducted by Dr. Susan Rotzinger, Mawahib O. Semeralul, and Paul C. Boutros, however comparative analysis and validation of Experiment Two were conducted by L.A.F. In Experiment One, both locomotor and sugar feeding behaviour were found to have normal distributions (Figures 7a and b), unlike the EPM open arm time and startle responses. Scores for the final day of sugar consumption testing in Experiment Two showed a similarly normal distribution when compared to Experiment One (Figure 8a). However, Experiment Two had more animals eating no or very little sugar on the testing day, where 20% (8/40 animals total) did not consume appreciable quantities of sugar during the testing period versus 10% (2/20 animals) in Experiment One. Figure 8d illustrates acquisition of a taste for sugar over four days preceding the test day. Note how the distribution of sugar consumption became more normalized over time, suggesting a Gaussian distribution for this behavioural phenotype.

Locomotion scores between Experiment One and Experiment Two also had similar Gaussian distributions. Locomotor crossovers were shown to be much greater in
Experiment One than in Experiment Two, however measurements were taken in different apparatus making comparison impossible except by relative conversions, which for this parameter is not possible.

Startle scores between the two experiments showed a bimodal distribution, where Experiment One rats startled less than Experiment Two rats. Rats undergoing startle testing in Experiment Two undertook it as a part of a prepulse inhibition paradigm, which included startle noise, prepulse alone, and prepulse + startle for two separate decibel levels of prepulse. There was no decrease in startle response (habituation) over each day of testing for Experiment Two rats (data not shown).

Figure 8a also shows EPM open-arm time from both experiments. More rats (63%, or 10/20 rats total) from Experiment One spent less than 10% of total time in the open arm of the EPM than rats in Experiment Two (50%, or 17/27 rats total). Rats from Experiment Two spent more time in the open arms of the EPM, a finding consistent with the higher percent total locomotion scores obtained in the locomotor boxes (see Figure 8b). This may be due to lower light in the testing facility, improving comfort levels of the animals in their novel environment, and may have also facilitated attrition of Experiment Two rats in EPM testing by disqualification due to falling off the EPM.

Figure 9 displays whole group mean score comparisons between Experiment One and Experiment Two test results across sugar feeding, average startle, EPM % time spent in the open arm, per cent total locomotion and locomotion crossovers. Very highly significant differences between means were determined for startle scores (p<0.0001), EPM % time spent in the open arm (p<0.0004), and for locomotion crossover parameters
(p<0.0001) using an unpaired, two-tailed, Student’s t-Test with appropriate correction for equal or unequal variances based on significant F-test results. Sugar feeding means were significantly different at p<0.049. No significant difference was seen between experimental groups for percent total locomotion.

Figure 10 shows statistical comparisons between measures in both Experiment One and Experiment Two, especially in individuals selected for array expression profiling. Sugar consumption was shown to be significantly lower for low-responding individuals in both Experiment One (p<0.0035) and Experiment Two (p<0.0008) when compared to their respective experiment high-responding counterparts. Similarly for percent total locomotion scores, high responding individuals in both Experiment One and the Experiment Two had statistically higher scores when compared to low responding individuals in the same experiment (Experiment One, p<0.0104; Experiment Two, p<0.0006). No statistical significance was seen between individuals of either high or low response category for percentage of time spent in the open arm of the EPM, or for startle scores. Significant differences in locomotion crossovers were seen (p<0.0024) between high and low responders in Experiment One. Figure 11 demonstrates the strength of association between the tests (sugar feeding and per cent total locomotion) selected for
Figure 7a. Behavioural histograms for Experiment One.

Histograms for sugar feeding shows relatively normal distribution. EPM open arm time histogram shows a skewed distribution towards lower response ranges, which was expected for EPM scores given the Lewis rat’s higher propensity to anxiety.
Figure 7b. Behavioural histograms for Experiment One.

Startle scores show a skewed distribution in this histogram, and locomotor counts were distributed somewhat normally.
Sugar consumption, % Total locomotion scores, locomotion crossovers, startle scores and EMP scores were compared for Experiments One and Two. Similar distributions were seen between Experiments One and Two for sugar consumption, locomotion, but completely divergent distributions were seen for locomotion crossovers and for startle scores. These latter two scores may differ because of a change in testing apparatus. %EPM in open arm showed a non-normal distribution for both Experiment One and Two, and a significant skew for Experiment Two to lower values (probably because of animals falling off of the platform). See all across Figures 8a-c.
Figure 8b. Histograms comparing Experiments One and Two.
Figure 8c. Histograms comparing Experiments One and Two.
Figure 8d. Sugar consumption from day of first exposure to test day for Experiment Two rats.

Shows the normalization of sugar consumption across all five days of exposure and demonstrates sucrose consumption acquisition. (n=40 rats)
Experiment One vs Two:
EPM % Open Arm

Experiment One vs Two:
Average Startle

Experiment One vs Two:
Sugar Feeding

Experiment One vs Two:
Locomotion

Experiment One vs Two:
Locomotion Crossovers

Figure 9. Whole group mean score comparisons between Experiment One and Two.

Significant differences in several scores were seen between both Experiment groups One and Two, indicating that the testing apparatus differences may have significantly contributed to behaviour response in the tests. Each test, except for locomotion, showed a significant (p<0.05) difference between Experiment One and Experiment Two scores.
Figure 10. Comparison of behaviour measures between Experiments One and Two for animals selected for array expression profiling.

Significant differences in scores between high and low groupings of individuals were seen for sugar consumption in both Experiment One and Experiment Two (One, p<0.0035; Two, p<0.0008, top row, left graph). Similarly significant differences were seen between high and low groups for both Experiments for % total locomotion scores (One, p<0.0006; Two, p<0.0104). No significant differences were seen for EPM scores, startle scores, or for Experiment Two high and low groups for locomotion crossover scores. Experiment One high and low groups showed significant differences in locomotion crossovers (p<0.0024), however the locomotion testing equipment was different in the two Experiments.
Figure 11. Pearson correlations between behaviour scores for Experiment One and Experiment Two

Experiment One (top row) shows low correlation (r) values with non-significant p-values. Experiment Two (bottom row) shows a weak correlation (r=0.1014, p=0.0452) between sugar feeding and total locomotion, and between startle and total locomotion (r=0.1181, p=0.0299). For array-selected animals, Experiment One revealed a significant correlation between high and low animals for sugar feeding vs locomotion scores (r=0.7667, p=0.0214, n=9) but not between sugar feeding and startle (r=-0.05, p=0.9116, n=9) or between startle and locomotion (r=-0.2500, p=0.5206, n=9). Experiment Two showed near significance between sugar feeding and locomotion scores (r=0.5879, p=0.0806, n=10) but not for sugar feeding vs startle (r=-0.1515, p=0.6821, n=10) or between startle and locomotion (r=0.4667, p=0.1786, n=10).
population sampling and subsequent microarray processing. There was no significant
correlation between the two sample groups in Experiment One (\(p=0.94\)) but there was a
weak negative correlation (\(r=-0.3184, r^2=0.1014, p=0.0452, n=40\)) between sugar feeding
and per cent total locomotion in the Experiment Two population.

4.2 Data analysis

Technical assistance and microarray data analysis was provided by Paul Boutros.
Unsupervised machine-learning (cluster analysis) was inappropriate for our experimental
design (Boutros and Okey, 2005). Instead, a formal statistical analysis was undertaken,
applying linear-modeling to identify the relationship between behavioural scores and
mRNA abundances. Please refer to Figure 12 for a flowchart explaining the analysis
methods for these experiments.

Summary

Scanned array images were visually inspected to verify that the arrays were free
of gross artifacts and to ensure spatial heterogeneity. Signal intensities were pre-
processed with the GC-RMA algorithm, which transforms the data into a log2-scale
(Irizarry et al., 2003). Spearman correlations between each the normalized values for each
ProbeSet on the array and the vectors of behavioural scores were calculated. Statistical
significance of Spearman’s rho (the probability that rho is significantly different from
zero) employed the method of Best & Edwards (Best and Roberts, 1975). To link the
correlations with the magnitude of effect, the behavioural scores were subjected to linear
modeling. The fitted models were subjected to empirical Bayes moderation of standard
error (Smyth, 2004) and an FDR adjustment (Storey and Tibshirani, 2003).
Experiment One was analyzed initially using a Signal-to-Noise method (described in Methods). Method 1, which resulted in a gene list from which three genes were selected for array confirmation by qRT-PCR. Experiment Two was subsequently conducted to attempt cross-experiment validation of behaviour with qRT-PCR. Genes were selected from Experiment One analysis (Method 1) to attempt to cross-experiment validate (see Table 4, 5a, 5b). Our validation of these genes found significant correlations for all scores, however there was no overlap between qRT-PCR validation and Experiment One measures (see Table 5a, 5b). Because of this, a second analysis method, Method 2, was implemented across both data sets (Experiment One and Experiment Two) due to advances in analytical processing in microarray analysis methodology. From this, two gene lists were derived, sorted, and a number of genes that overlapped between the two Experiments and had adequate significance and expression differences between high and low groups of individuals to be preferentially selected for validation (see Table 2b). From this list, four genes were selected for further validation, and there was one overlap (Snx27) between both generated lists (Table 2b). qRT-PCR was conducted and array/behaviour validation undertaken (Table3,4).
FDR adjustment is an important correction to remove the potential bias of multiple hypothesis testing. The coefficients and p-values representing the association between expression and scores were extracted, resulting in linear model fits. The maximal fold-change was calculated for each ProbeSet (in log₂-space) based on the linear modeling coefficients and the behavioural score range. The correlation and linear-modeling analyses were combined and ProbeSets were associated with updated genomic annotation using the NetAffx database. Two separate analytical methods were used for ProbeSet selection for validation due to the advancement of analytical methodology development over the time points when Experiment One and Experiment Two were conducted, and are outlined in Chapter 3.3, 3.4 and 3.8.

4.2.1 Experiment One and Experiment Two microarray analysis, gene list preparation

4.2.1.1 SNR analysis

Two separate methods of analysis were undertaken to determine the amount of overlap in array data between Experiment One and Experiment Two (Figure 13, 14) due to the improvement of analysis paradigms between the time of Experiment One and Experiment Two (>1 yr). As genes for confirmation for Experiment Two were initially selected for validation from the SNR-generated gene list from Experiment One (Table 2a) for across-batch qRT-PCR validation of behaviour by correlation, and array and behaviour validation from the gene list generated from Experiment Two (Tables 2b and 2c), the two separate methods beyond SNR analysis are outlined below (Methods One
and Two), and delineate their respective outcomes for array validation in this section. Subsequently behavioural validation using qRT-PCR will be discussed.

4.2.1.2 Method One

Since the two experiments employed different array platforms GeneChip platforms (RAE 230A for Experiment One and RAE 230 2.0 for Experiment Two), analysis of all possible ProbeSets for overlap was required. The RAE230A GeneChip represents a subset of genes queried on the RAE230 2.0 array. Because of this, 7555 ProbeSets were initially sampled. This indicated the number of ProbeSets showing statistically significant differences in expression from background signal between Experiment One and Experiment Two for all four testing measurements (startle, locomotion, elevated plus maze and sugar feeding, see Figure 13a). The overlap in expression on each Experimental set of arrays was determined using 15,923 ProbeSets from both experiments, the number of distinct ProbeSets on the RAE230A, and which is a subset of the 31,100 ProbeSets found on the RAE230 2.0 array. Figure 13a (7,555 ProbeSets total) describes the number of overlapping ProbeSets between the two Experiments where uncorrelated ProbeSets (i.e. ProbeSets with \( p_{\text{adjusted}} > 0.05 \)) and ProbeSets with very-low signal spots are removed (~7000 ProbeSets). Figure 13b describes a similar analysis including all ProbeSets in the subset, with genes that may not be expressed at high levels but which may yet have some impact on phenotype; Figure 13c describes the overlap of ProbeSets annotated with enriched GO terms. The number of overlapping ProbeSets between Experiment One and Experiment Two were very small, and did not overlap between behavioural tests.
Figure 13. Experiment One and Two microarray ProbeSet overlap using analysis Method One

Analysis between experiments was conducted to determine if any gene expression overlap was seen in the two population samples. Number of ProbeSets displayed indicate number of ProbeSets viable for quantitation after processing for low signal and background, using analysis Method One. Left-hand circles represent Experiment One, right-hand circles, Experiment Two.

a) Shows comparison only of the RAE230a subset of genes between Experiment One arrays and Experiment Two arrays using high gene expression filtering, the left-hand number indicates the number of ProbeSets in the intersection of both Experiment gene lists, the second number indicates the number of ProbeSets one would expect to appear in this analysis by chance alone.

b) Shows the comparison of the subset of genes available to both array sets using no filtering, and describes ProbeSet overlap between the two experiments using enriched GO terms. The table shows fold enrichment per behavioural test, per filter method of evaluation.
Figure 14. Venn diagrams showing overlapping ProbeSets between experimental measures in Experiment One and Experiment Two using Method 2 of analysis

a) ProbeSet overlap between experimental measures within Experiment One and Experiment Two. b) ProbeSet overlap between Experiment One and Experiment Two for three individual measures. c) ProbeSet overlap between sugar feeding and locomotion for Experiment One and Experiment Two. Venn diagrams were created using “Venny”. Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.
<table>
<thead>
<tr>
<th>ProbeSet</th>
<th>Mean</th>
<th>SNR</th>
<th>UniGene Cluster</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1387909_at</td>
<td>10.2</td>
<td>-8.9</td>
<td>Rn.11316</td>
<td>Abl-interactor 2</td>
</tr>
<tr>
<td>1371931_at</td>
<td>9</td>
<td>-7.5</td>
<td>Rn.27575</td>
<td>General transcription factor II I</td>
</tr>
<tr>
<td>1393418_at</td>
<td>7.6</td>
<td>6.6</td>
<td>Rn.74047</td>
<td>Tropomodulin 2</td>
</tr>
<tr>
<td>1369332_a_at</td>
<td>7.2</td>
<td>5.4</td>
<td>Rn.10799</td>
<td>Regulating synaptic membrane exocytosis 1</td>
</tr>
<tr>
<td>1370002_at</td>
<td>7.2</td>
<td>-4.6</td>
<td>Rn.64481</td>
<td>Rho guanine nucleotide exchange factor (GEF) 1</td>
</tr>
<tr>
<td>1390447_at</td>
<td>6.4</td>
<td>4.4</td>
<td>Rn.10951</td>
<td>Syntaxin 3</td>
</tr>
<tr>
<td>1374235_at</td>
<td>6.8</td>
<td>4.1</td>
<td>Rn.24428</td>
<td>Down syndrome critical region gene 1-like 1</td>
</tr>
<tr>
<td>1373462_at</td>
<td>6.4</td>
<td>-2.6</td>
<td>Rn.3939</td>
<td>Embryonic ectoderm development (predicted)</td>
</tr>
<tr>
<td>1387036_at</td>
<td>6.2</td>
<td>-2.4</td>
<td>Rn.19727</td>
<td>Hairy and enhancer of split 1 (Drosophila)</td>
</tr>
<tr>
<td>1371806_at</td>
<td>6.9</td>
<td>-2.3</td>
<td>Rn.17134</td>
<td>DiGeorge syndrome critical region gene 8 (predicted)</td>
</tr>
<tr>
<td>1368572_a_at</td>
<td>9.8</td>
<td>-2.2</td>
<td>Rn.9840</td>
<td>Glutamate receptor, ionotropic, N-methyl D-aspartate 1</td>
</tr>
<tr>
<td>1368650_at</td>
<td>7.7</td>
<td>-2.1</td>
<td>Rn.2398</td>
<td>TGFB inducible early growth response</td>
</tr>
<tr>
<td>1368261_at</td>
<td>9</td>
<td>-2.1</td>
<td>Rn.10926</td>
<td>Neurexin 3</td>
</tr>
<tr>
<td>1398353_at</td>
<td>6.9</td>
<td>2</td>
<td>Rn.15760</td>
<td>SAR1a gene homolog 1</td>
</tr>
</tbody>
</table>

Table 2a. SNR analysis of Experiment One

Results of a signal-to-noise ratio analysis between rats in the high and low groups. An SNR analysis (see Methods, section 3.3.1) was run for each ProbeSet on the array; selected results are given in this table. The Mean gives the average signal intensity (generally proportional to mRNA level) across both groups of animals. The SNR gives the ratio of signal to noise, with positive values indicating higher expression in the “high” group and negative values indicating higher expression in the “low” group.
<table>
<thead>
<tr>
<th>Cluster</th>
<th>Low</th>
<th>High</th>
<th>P-Value</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn.27575</td>
<td>9.2</td>
<td>8.8</td>
<td>0.00</td>
<td>general transcription factor II l</td>
</tr>
<tr>
<td>Rn.64481</td>
<td>7.3</td>
<td>7.0</td>
<td>0.00</td>
<td>lsc protein</td>
</tr>
<tr>
<td>Rn.44871</td>
<td>6.9</td>
<td>6.5</td>
<td>0.00</td>
<td>dihydropyrimidinase-related protein</td>
</tr>
<tr>
<td>Rn.37325</td>
<td>7.7</td>
<td>7.4</td>
<td>0.01</td>
<td>Rattus norvegicus transcribed sequences</td>
</tr>
<tr>
<td>Rn.82696</td>
<td>7.0</td>
<td>7.4</td>
<td>0.01</td>
<td>cryptochrome 2 (photolyase-like)</td>
</tr>
<tr>
<td>Rn.24871</td>
<td>9.1</td>
<td>8.8</td>
<td>0.01</td>
<td>phosphatidylinositol binding clathrin assembly protein</td>
</tr>
<tr>
<td>Rn.109</td>
<td>8.8</td>
<td>8.3</td>
<td>0.02</td>
<td>Rattus norvegicus similar to hydrophilic protein (LOC309629), mRNA</td>
</tr>
<tr>
<td>Rn.10249</td>
<td>6.0</td>
<td>6.4</td>
<td>0.03</td>
<td>cytochrome b5, outer mitochondrial membrane isoform</td>
</tr>
<tr>
<td>Rn.19727</td>
<td>6.4</td>
<td>5.9</td>
<td>0.03</td>
<td>hairy and enhancer of split 1 (Drosophila)</td>
</tr>
<tr>
<td>Rn.18761</td>
<td>7.0</td>
<td>6.3</td>
<td>0.03</td>
<td>Rattus norvegicus transcribed sequences</td>
</tr>
<tr>
<td>Rn.7534</td>
<td>10.3</td>
<td>9.9</td>
<td>0.03</td>
<td>Rattus norvegicus transcribed sequence with weak similarity to protein sp:O94772 (H. sapiens) LY6H_HUMAN Lymphocyte antigen Ly-6H precursor</td>
</tr>
<tr>
<td>Rn.47579</td>
<td>8.0</td>
<td>7.6</td>
<td>0.04</td>
<td>Rattus norvegicus transcribed sequences</td>
</tr>
<tr>
<td>Rn.2398</td>
<td>8.0</td>
<td>7.3</td>
<td>0.05</td>
<td>TGFB inducible early growth response</td>
</tr>
<tr>
<td>Rn.103672</td>
<td>8.0</td>
<td>7.5</td>
<td>0.05</td>
<td>Rattus norvegicus transcribed sequence with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Operon Repressor Bound To 21-Base Pair Symmetric Operator Dna, Alpha Carbons Only</td>
</tr>
<tr>
<td>Rn.37500</td>
<td>7.5</td>
<td>6.8</td>
<td>0.05</td>
<td>homer, neuronal immediate early gene, 1</td>
</tr>
<tr>
<td>Rn.9775</td>
<td>7.6</td>
<td>7.0</td>
<td>0.06</td>
<td>HIF-1 responsive RTP801</td>
</tr>
<tr>
<td>Rn.3502</td>
<td>5.6</td>
<td>6.2</td>
<td>0.07</td>
<td>Rattus norvegicus similar to cAMP-dependent protein kinase, beta-2-catalytic subunit (PKA C-beta-2) (LOC310986), mRNA</td>
</tr>
<tr>
<td>Rn.3239</td>
<td>6.7</td>
<td>6.3</td>
<td>0.07</td>
<td>V-1 protein</td>
</tr>
<tr>
<td>Rn.1410</td>
<td>6.8</td>
<td>7.3</td>
<td>0.11</td>
<td>Rattus norvegicus similar to evecdin-2 (LOC301337), mRNA</td>
</tr>
<tr>
<td>Rn.59831</td>
<td>5.4</td>
<td>5.9</td>
<td>0.15</td>
<td>Rattus norvegicus transcribed sequences</td>
</tr>
<tr>
<td>Rn.30593</td>
<td>7.2</td>
<td>6.7</td>
<td>0.19</td>
<td>Rattus norvegicus transcribed sequences</td>
</tr>
</tbody>
</table>

**Table 2b. Sample gene lists for microarray validation Experiment One and behaviour validation Experiment Two**

Gene lists presented in this figure are representative of the actual gene lists used to select validation candidates, but do not include all selected genes. Partial candidate gene-lists from Experiment One (Table 2B) and Experiment Two (Table 2c) microarray data are shown and were generated using analysis Method One. Highlighted genes were those selected for validation of the original array and coincide with the original SNR analysis.
<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Symbol</th>
<th>Loco max fold change</th>
<th>UniGene ID</th>
<th>Gene Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1391478_at</td>
<td>Znf532_predicted</td>
<td>3.41E-03</td>
<td>0.23</td>
<td>zinc finger protein 532 (predicted)</td>
</tr>
<tr>
<td>1371203_at</td>
<td>Znf291</td>
<td>0.04</td>
<td>0.26</td>
<td>zinc finger protein 291</td>
</tr>
<tr>
<td>1371471_at</td>
<td>Zfp499_predicted</td>
<td>0.01</td>
<td>0.95</td>
<td>zinc finger protein 499 (predicted)</td>
</tr>
<tr>
<td>1370865_at</td>
<td>Zeb1</td>
<td>0.05</td>
<td>1.22</td>
<td>zinc finger 1-box binding homeobox 1</td>
</tr>
<tr>
<td>1373399_at</td>
<td>Wnt6</td>
<td>0.04</td>
<td>0.7</td>
<td>Wnt 6 receptor domain</td>
</tr>
<tr>
<td>1373955_at</td>
<td>Ymnd4</td>
<td>2.84E-03</td>
<td>0.56</td>
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</tr>
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<td>1386797_at</td>
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<td>1.18</td>
<td>TBC1 domain family, member 14</td>
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<tr>
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<td>0.74</td>
<td>TAR DNA binding protein</td>
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<tr>
<td>1371023_at</td>
<td>Zeb2</td>
<td>0.01</td>
<td>0.91</td>
<td>zinc finger 1-box binding homeobox 2</td>
</tr>
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<td>1383766_at</td>
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<td>0.57</td>
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<tr>
<td>1399024_at</td>
<td>Scyl1</td>
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<td>1.28</td>
<td>SCY1-like 1 (S. cerevisiae)</td>
</tr>
<tr>
<td>1370169_at</td>
<td>Retn4r</td>
<td>0.03</td>
<td>0.51</td>
<td>reticulon 4 receptor</td>
</tr>
<tr>
<td>1388582_at</td>
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<td>0.17</td>
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<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
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<td>PRF1 perforin</td>
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<td>0.01</td>
<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
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<td>1.2</td>
<td>PRF3 perforin</td>
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<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
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<td>1372246_at</td>
<td>Prf4</td>
<td>0.01</td>
<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
<tr>
<td>1389058_at</td>
<td>Prf1</td>
<td>0.04</td>
<td>1.2</td>
<td>PRF1 perforin</td>
</tr>
<tr>
<td>1372365_at</td>
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<td>0.01</td>
<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
<tr>
<td>1372246_at</td>
<td>Prf3</td>
<td>0.01</td>
<td>0.48</td>
<td>interleukin 4 receptor</td>
</tr>
<tr>
<td>1389058_at</td>
<td>Prf4</td>
<td>0.04</td>
<td>1.2</td>
<td>PRF4 perforin</td>
</tr>
</tbody>
</table>

Table 2c. Sample gene lists for microarray validation Experiment Two and behaviour validation Experiment One and Two.

The ProbeSets shown in this gene list were selected by comparing gene lists from both Experiment One and Experiment Two which were generated by analytical Method Two, and which had greater overlap between Experiments than seen in Method One (Figure 12 and 13). Genes highlighted are those selected for RT-PCR validation based on p-values in relation to linear modeling for array data vs. locomotion scores and sugar feeding scores. One gene overlapped from Method One analysis, Snx27.
4.2.1.3 Method Two

This method of analysis involved re-analysis of both sets of data (Experiment One and Experiment Two) by the same method, as described in section 3.7. The results from each experiment were subjected to a threshold of $p_{\text{adjusted}} < 0.05$ and the overlap amongst selected genes was compared between the two experiments (Figure 14).

A gene list was produced for Experiment One using SNR analysis, from which three candidate genes were selected for validation: Hes1, Homer1, and Tieg (also known as Klf10) (Table 2b), using fold change differences, and a sufficiently low p-value ($<0.05$) of values extracted in log$_2$ space. These three genes were selected to validate Experiment One, and Hes1 and Homer1 had statistically significant or nearly statistically significant differences between behavioural groups, consistent with the array data (Table 3c).

Additional gene lists were generated for Experiment One and Two using Method One and Method Two analysis approaches (see Table 2b and Table 2c, respectively). Selection criteria included relatively high absolute differences in expression values, significance of correlation with a behavioural score ($p<0.05$), and that the gene was not one of the following: non-coding sequence, predicted sequence, or an unnamed sequence without annotation, since pursuing these transcripts would be less cost-effective. These limitations were put in place to facilitate RT-PCR validation. Interestingly, several genes arose during the analyses that are related to a spectrum of psychiatric diseases, some of which may be developmentally, metabolically, or structurally linked.
4.2.2 Microarray validation for Experiment One and Experiment Two

4.2.2.1 Experiment One array validation using SNR analysis as selection criteria

Validation was conducted in three ways, based on current analysis methodology. Genes selected from Table 2a and generated via the older, less statistically strong SNR analysis method, were initially validated for Experiment One. Hes1 was the only gene that resulted in any correlation between array, RT-PCR and behaviour (locomotion scores) (Figure 15). However, Homer1 RT-PCR expression data validated the array ProbeSet assayed using Spearman’s Rank correlation analysis (Rho = -1, p = 0.0001). Hes1 just missed significance with Rho = 0.7714, p = 0.0724, n=6 (Table 3b). Tieg/Klf10 did not show array validation using RT-PCR. Hes1 expression was, by original analysis methods, shown to be significantly correlated with locomotion behaviour scores (Figure 15), however Homer1 and Tieg/Klf10 RT-PCR and array data had even lower correlations when compared to behavioural data, indicating a great deal of noise between the two methods of expression analysis. 14 ProbeSets were identified using this method of analysis, and 12 were selected for validation by RT-PCR, three of which were validated during for Experiment One array set, and were subsequently used to attempt to validate array data for Experiment Two.
Figure 15. Correlation of qRT-PCR and ProbeSet values with locomotion score for Hes-1 from Experiment One

To validate that the array study, mRNA levels were measured by real-time PCR. Both array- and PCR-derived expression estimates of *Hes1* are plotted against Locomotion test-scores. Array and PCR values were normalized as described in methods, then scaled onto separate 0-100 scales for visualization. The two assays recapitulate nearly identical relationships between *Hes1* expression and Locomotion test-scores, with similar slopes and Y-intercepts.
4.2.2.2 Experiment One and Two validation by Method One
selection criteria

Figures 13 (a, and b) show overlap between ProbeSet lists for both Experiments derived from this method of analysis, using both high expression filtering (13a), and no filtering (13b) to determine the best selection criteria for transcript validation. High expression filtering significantly reduced the number of like ProbeSets between the two Experiments for every behavioural score, and rendered the analysis not statistically significant (see Table in Figure 13). Candidate genes met the criteria of having a respectable level of average expression for the group of ProbeSets sampled, for example, an average expression level which is greater than six. Collapsing ProbeSets into functional groups using GO enrichment analysis did not lead to improved overlap between the two experiments.

4.2.2.3 Experiment One and Two validation by Method Two
selection criteria

Figure 14a, b, and c show overlap between ProbeSet lists derived from Method Two analysis of both Experiments. Overlap within experiments was very low for Experiment One, with the maximal number of ProbeSets overlapping between behavioural scores at 1.1% for locomotion and startle, 1.3% for locomotion and sugar feeding, and 0.53% for sugar feeding and startle (Figure 14a). Experiment Two showed improved overlap between behavioural scores, although with a greater number of ProbeSets sampled (sugar feeding: locomotion = 8.4% ProbeSet overlap; sugar feeding: startle = 0.4% ProbeSet overlap; locomotion: startle = 10.4% ProbeSet overlap, all three behavioural groups = 0.4% ProbeSets overlapping (Figure 14a). One might expect that behaviours rooted in similar neurotransmitter pathways (i.e. dopaminergic pathway for sugar feeding and locomotion) might share a higher quantity of overlap of ProbeSets than behaviours resulting from very different neurotransmitter pathways. Most importantly,
Figure 14b shows overlap between Experiments for each behaviour category, with locomotion having produced an overlap of 1.5% of all ProbeSets, sugar feeding producing an overlap of 0.5% of ProbeSets, and startle producing an overlap of 2.5% of ProbeSets sampled across both Experiment One and Experiment Two, showing that very few of the transcripts were associated with each of the four complex behavioural phenotypes measured overlapped, and indicating that the four behavioural experiments were far enough apart transcriptomically as to act as separate experiments, or that the differences in behaviour are driven by non-coding RNA. Table 2c delivers a good example of genes remaining within the selection criteria, before elimination of unnamed or only predicted sequences. 53 ProbeSets total were generated using this Method of analysis, and four transcripts were selected for validation using RT-PCR.

4.2.3 Array validation using Method One and Method Two gene list

ProbeSets for validation with RT-PCR

Fluorescence data collected during the log phase of amplification (i.e. $\Delta Rn$) during PCR cycling were plotted against respective cycle number. ABI7500 analysis software calculates $\Delta Rn$ using the equation:

$$\Delta Rn = Rn^+ - Rn^-$$

Where $Rn^+ = \text{fluorescence emission of product at each timepoint}$ and

$Rn^- = \text{fluorescence emission of baseline measurement}$

When plotted as described above these values created the amplification plot for each sample, from which an average Te and standard deviation were calculated for each sample. Results were exported to Microsoft Excel for linear regression analysis.
Standard curves were prepared for each gene tested, in addition to each endogenous control used for comparison, and for each sample the quantity of endogenous reference and target gene were extrapolated from their respective standard curves. Experimental samples were normalized to an arbitrarily chosen sample (referred to as the 1x sample, usually the first sample), and each of the target gene values were divided by this calibrator value to generate a normalized value. The normalized value was subsequently compared to a mean relative dilution of the reference gene. Normalization was conducted to control for experimental confounders such as different molecular quantities of RNA, or unequal efficiency of reverse transcription and PCR amplification. Linear regression analysis was conducted using the “least squares” method to evaluate standard curves. Relative dilutions are as described in the equation below:

$$\text{Relative dilution of target gene} = \frac{\text{Mean of relative dilution of Target Gene}}{\text{Mean of relative dilution of Reference Gene}}$$

Table 3b shows validation results by RT-PCR, for both Experiment One and Experiment Two, selected by SNR analysis method. Homer1 showed significant correlation for array and RT-PCR values, Hes1 nearly reached significance ($p=0.0724$, two tailed, Spearman’s Rho). Genes were selected for validation using the original SNR analysis method gene list and can be seen in Table 3a. It is clear from these tables that the genes show significance when correlated with array data from Experiment Two (Array #2 in the Table), but do not at all correlate with array data from Experiment One (Array #1), suggesting that Experiment Two does not replicate Experiment One.
Table 3b includes genes selected from Experiment Two arrays alone (i.e. Fblx10, Nfib, Bbs2, Smarca4, and Snx27) using Method One for analysis. These ProbeSets, processed statistically in the same manner as those in Table 3a, resulted in very few ProbeSets being validated using correlative analysis, with one of Smarca4 ProbeSets being the only ProbeSet to reach statistical analysis. One other ProbeSet for Smarca4 and one for Nfib were nearly significant (p=0.0816 and p=0.0984, respectively, see Table 3c, Experiment Two), but no other ProbeSets selected from this analysis method were validated using RT-PCR.

Method Two of array analysis produced an overlapping gene list between Experiment One and Experiment Two (Table 2c) from which one ProbeSet overlapped with Method One analytical gene list creation (Snx27). Four other ProbeSets correlated with the following genes were selected for validation: Ppp1r1a, Gabrg1, Cort, and Cdk5. Table 3c shows that none of these genes were validated using Spearman’s Rho correlation.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>ProbeSet</th>
<th>Spearman's rho &amp; p-value</th>
<th>Locomotion</th>
<th>Sugar Feeding</th>
<th>Startle</th>
<th>EPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Array #1</td>
<td>Array #2</td>
<td>PCR</td>
<td>Array #1</td>
</tr>
<tr>
<td>Abi2</td>
<td>1387090_at</td>
<td>rho -0.085</td>
<td>0.491</td>
<td>-0.076</td>
<td>0.855</td>
<td>0.406</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.828</td>
<td>0.149</td>
<td>0.641</td>
<td>0.828</td>
<td>0.240</td>
</tr>
<tr>
<td>Cry2</td>
<td>1369446_at</td>
<td>rho 0.267</td>
<td>-0.018</td>
<td>0.198</td>
<td>0.267</td>
<td>0.067</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.493</td>
<td>0.973</td>
<td>0.221</td>
<td>0.493</td>
<td>0.851</td>
</tr>
<tr>
<td>Dscr1l1</td>
<td>1374235_at</td>
<td>rho -0.200</td>
<td>-0.055</td>
<td>-0.118</td>
<td>-0.233</td>
<td>-0.321</td>
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<tr>
<td>p-value</td>
<td></td>
<td>0.613</td>
<td>0.892</td>
<td>0.475</td>
<td>0.552</td>
<td>0.368</td>
</tr>
<tr>
<td>Grin1</td>
<td>1368572_a_at</td>
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<td>-0.040</td>
<td>0.133</td>
<td>-0.345</td>
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<tr>
<td>p-value</td>
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<td>1.000</td>
<td>0.828</td>
<td>0.149</td>
<td>0.641</td>
<td>0.828</td>
</tr>
<tr>
<td>Hbp1</td>
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<td>0.234</td>
<td>-0.479</td>
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<tr>
<td>p-value</td>
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<td>0.339</td>
<td>0.544</td>
<td>0.166</td>
</tr>
<tr>
<td>Homer1</td>
<td>1368319_a_at</td>
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<td>0.250</td>
<td>0.200</td>
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<td>p-value</td>
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<td>0.076</td>
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<td>Nrxn3</td>
<td>1368261_at</td>
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<td>p-value</td>
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<td>0.772</td>
<td>0.500</td>
<td>0.521</td>
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</tr>
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<td>Picalm</td>
<td>1373877_at</td>
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<td>0.103</td>
<td>0.333</td>
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</tr>
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<td>p-value</td>
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<td>0.772</td>
<td>0.036</td>
<td>0.795</td>
<td>0.892</td>
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<td>Rims1</td>
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<td>0.367</td>
<td>0.373</td>
<td>0.103</td>
</tr>
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<td>p-value</td>
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<td>0.569</td>
<td>0.772</td>
<td>0.020</td>
<td>0.323</td>
<td>0.772</td>
</tr>
<tr>
<td>Stx3</td>
<td>1390447_at</td>
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<td>-0.333</td>
<td>0.321</td>
<td>0.318</td>
<td>-0.139</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.898</td>
<td>0.349</td>
<td>0.444</td>
<td>0.404</td>
<td>0.707</td>
</tr>
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<td>Klf10</td>
<td>1368650_at</td>
<td>rho 0.100</td>
<td>-0.164</td>
<td>-0.217</td>
<td>-0.167</td>
<td>-0.297</td>
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<td>p-value</td>
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<td>0.810</td>
<td>0.657</td>
<td>0.178</td>
<td>0.678</td>
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</tr>
<tr>
<td>Tmod2</td>
<td>1393418_at</td>
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<td>0.374</td>
<td>0.150</td>
<td>0.188</td>
</tr>
<tr>
<td>p-value</td>
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<td>0.843</td>
<td>0.240</td>
<td>0.018</td>
<td>0.708</td>
<td>0.596</td>
</tr>
</tbody>
</table>

Table 3a. Microarray and Real-time RT-PCR confirmation of gene transcripts

Highlighted values are statistically significant and describe the molecular test in Spearman’s correlation with the behaviour score. There was little validation between the either of the Experimental groups array data and the PCR results from Experiment Two.
### Experiment One: Correlation of Array ProbeSet with qRT-PCR data of High and Low Animals (n=6)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>ProbeSet</th>
<th>Pearson r-squared</th>
<th>p-value</th>
<th>Rho</th>
<th>p-value, exact, two tailed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hes1</td>
<td>1387036_at</td>
<td>0.5046</td>
<td>0.1137</td>
<td>0.7714</td>
<td>0.0724</td>
</tr>
<tr>
<td>Homer1</td>
<td>1370656_a_at</td>
<td>0.8485</td>
<td>0.0091</td>
<td>-1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tieg/Klf10</td>
<td>1368650_at</td>
<td>0.09179</td>
<td>0.5594</td>
<td>0.08571</td>
<td>0.8717</td>
</tr>
</tbody>
</table>

### Experiment Two: Correlation of Array ProbeSet with qRT-PCR data for High and Low animals (n=10)

(Absolute Quantitative Array Value)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>ProbeSet</th>
<th>Rho</th>
<th>Rho-squared</th>
<th>p-value, exact, two tailed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fblx10</td>
<td>1393101_at</td>
<td>0.4909</td>
<td>0.241</td>
<td>0.1548</td>
</tr>
<tr>
<td>Nfib</td>
<td>1388167_at</td>
<td>0.4545</td>
<td>0.2066</td>
<td>0.19112</td>
</tr>
<tr>
<td></td>
<td>1371202_a_at</td>
<td>-0.2485</td>
<td>0.0618</td>
<td>0.4888</td>
</tr>
<tr>
<td>Bbs2</td>
<td>1368509_at</td>
<td>0.153</td>
<td>0.0106</td>
<td>0.777</td>
</tr>
<tr>
<td>Smarca4</td>
<td>1375469_at</td>
<td>-0.6364</td>
<td>0.405</td>
<td>0.0479</td>
</tr>
<tr>
<td></td>
<td>1373565_at</td>
<td>0.3333</td>
<td>0.1111</td>
<td>0.3466</td>
</tr>
<tr>
<td>Snx27</td>
<td>1385011_at</td>
<td>-0.5758</td>
<td>0.3315</td>
<td>0.0816</td>
</tr>
<tr>
<td>Ppp1r1a</td>
<td>1383191_at</td>
<td>0.2242</td>
<td>0.0503</td>
<td>0.5367</td>
</tr>
<tr>
<td>Cort</td>
<td>1387337_at</td>
<td>0.1273</td>
<td>0.0162</td>
<td>0.733</td>
</tr>
<tr>
<td>Gabrg1</td>
<td>1387706_at</td>
<td>0.0303</td>
<td>0.0009</td>
<td>0.946</td>
</tr>
<tr>
<td>Cdk5</td>
<td>1369089_at</td>
<td>-0.3091</td>
<td>0.0955</td>
<td>0.3869</td>
</tr>
</tbody>
</table>

Table 3b. Correlation of array ProbeSets (absolute quantitative array values) with RT-PCR data from Method One and Two

A) Genes selected for confirmation in Experiment One using SNR analysis. Homer1 was significant both in Pearson and Spearman’s Rho testing. Hes1 was nearly significant by Spearman Rank correlation analysis.

B) Genes selected for confirmation in Experiment Two. The top five genes (Fblx10 to Snx27) are from Method One analysis, the bottom four (Ppp1r1a to Cdk5) from Method Two. Snx27 overlapped both methods of analysis. Several genes had multiple ProbeSets.

Highlighted genes values were near or achieving statistical significance via Spearman’s Rank Correlation. Italicized results are statistically significant. Spearman test alone was conducted because of increased stringency and removal of the assumption of normalcy in the sample.
4.2.4  Behaviour Validation using RT-PCR

4.2.4.1 Validation of behavioural difference in Experiment One using RT-PCR

ProbeSets selected using SNR analysis method, Method One and Method Two were selected for array validation and subsequent behaviour analysis and validation in Experiment Two animal data. Table 4 outlines Spearman correlations of RT-PCR and behaviour scores for four separate scores measured during Experiment Two (excluding EPM scores). Genes highlighted and italicized indicate statistically significant Spearman correlation between RT-PCR and behaviour score and include Abli-2, Cox6c1, Dscrg1, Grin1, GTFII-I, Hes1, Homer1, Neurexin 3, PBCAP, RSME, Syx3a, Tropo-2, Bbs2, Gabrg1, Nfib and Ppp1r1a (see Table 4 for rho and p-values). Figure 16 provides examples for statistically significant correlations between RT-PCR and behaviour scores for locomotion and sugar consumption. Other genes (see Figure 4, highlighted, non-italicized) showed near significant correlations (just >0.05) between behaviour and RT-PCR expression.
Validation of behaviour was conducted both across the high and low animals (n=10) and across the whole sample (n=40) to attempt to determine whether false positives were being indicated.

Genes are listed in chronological order: Abli-2 through Tropo-2 were selected via Method One of analysis, Bbs2 through Snx27 through Method One, and Garbg1 through Cort through Method Two.  Note that boxes highlighted indicate near or statistical significance (italicized boxes indicate statistical significance in the Spearman correlation between RT-PCR and behaviour score across each group of animals), p-values were exact for array animals (n=10) and of Gaussian approximation with the whole sample (n=40).
Figure 16. Correlation of RT-PCR expression vs. behaviour scores

Pearson correlations between selected gene transcript expression and behaviour scores with which they show significant statistical correlation (from Table 4).
4.2.5 Endogenous Controls

Endogenous controls are used to assess the efficiency of hybridization assays as well as the quality of RNA samples on GeneChip expression arrays. Internal controls on the Affymetrix chips include hybridization controls (bioB, bioC, bioD, and cre) in addition to internal endogenous control genes (GAPDH, β-Actin, hexokinase on the RAE230 –both sets). The ideal endogenous control should remain consistent with minimal variations in expression levels across all treatment groups and within each individual sample.

**Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)**

GAPDH was selected as an endogenous control due to the heterogeneity and consistent high relative level of expression seen across all three control ProbeSets on the array (Figure 41) obtained by a default normalization method (scaling) to allow comparison.

Figure 41. GAPDH expression on Affy RAE230 2.0 GeneChip Array. The expression profile of endogenous control, GAPDH as represented by three separate ProbeSets, un-averaged.
between separate arrays in the experiment. Expression levels of B-actin and Hexokinase showed considerable variability between the three ProbeSets, and were expressed at less absolute quantities than GAPDH. GAPDH appeared to be the most stable and therefore was chosen as the control gene. The standard curve for GAPDH was used for the relative quantification of gene expression in regards to the selected target genes (i.e. Grin1, etc.) The “least squares” method was used to conduct linear regression analysis.

**Beta-Actin (B-actin)**

Alternately, we recognize that housekeeping/reference genes may not be adequate on their own to be used as a sole reference due to fluctuations within tissues based on treatments or even variabilities within highly differentiated tissues, such as brain (Coulson et al., 2008).
The practice of normalizing housekeeping gene expression is relatively new, and involves the generation of the geometric mean between more than one housekeeping gene’s RT-PCR measurements for each sample. This should smooth any large incidences of variability seen between treatment groups, tissue areas, disease state, and experimental conditions, and should produce a more stable measurement of normalization than using any single housekeeping gene (Vandesompele et al., 2002). As such, we decided to conduct this normalization on the final five genes we assayed using Method Two of analysis on Experiment Two RNA samples. Figure 43 shows that there is slightly more variability in B-actin expression on average between the three array ProbeSets than seen with GAPDH, although both, after scaling, almost overlapped in their absolute expression levels. As such, the geometric mean was taken of RT-PCR levels measured for each individual sample in Experiment Two for both GAPDH and for B-actin and applied to the final set of validation analysis.

Figure 44 provides a standard curve which showed less congruence between regression and actual RT-PCR values measured with a less tight fit (<0.99) than seen for GAPDH. However, the primer efficiency for this assay was greater than for GAPDH alone, and the geometric mean likely helped control for variability based on these differences.
4.2.6 Primer efficiencies

Primer efficiencies were calculated for all RT-PCR reactions using the formula from Wacker and Godard (2005):

\[
\text{Reaction efficiency} = \frac{10^{(-1/m)}}{2} \times 100
\]

Where \( m \) = slope of the log dilution standard curve using line of best fit

Calculation results are shown in Table 5.

Table 5. Primer efficiencies for RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>% Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abli-2</td>
<td>88.3</td>
</tr>
<tr>
<td>Cox6c1</td>
<td>132.8</td>
</tr>
<tr>
<td>Cry2</td>
<td>87.9</td>
</tr>
<tr>
<td>Dscr1</td>
<td>89.2</td>
</tr>
<tr>
<td>Grin1</td>
<td>105.9</td>
</tr>
<tr>
<td>GTFII-I</td>
<td>93.5</td>
</tr>
<tr>
<td>Hbp1</td>
<td>94.3</td>
</tr>
<tr>
<td>Hes1</td>
<td>94.6</td>
</tr>
<tr>
<td>Homer1</td>
<td>92.7</td>
</tr>
<tr>
<td>Neurexin3</td>
<td>84.7</td>
</tr>
<tr>
<td>PBCAP/Picalm</td>
<td>91.3</td>
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<tr>
<td>RSME/Rims</td>
<td>87.1</td>
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<td>Syx3a/Stx3</td>
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<tr>
<td>Fbxl10</td>
<td>108.7</td>
</tr>
<tr>
<td>Snx27</td>
<td>91.6</td>
</tr>
<tr>
<td>Smarca4</td>
<td>83.8</td>
</tr>
<tr>
<td>Nfib</td>
<td>86.0</td>
</tr>
<tr>
<td>Gabrg1</td>
<td>83.9</td>
</tr>
<tr>
<td>Cdk5</td>
<td>82.1</td>
</tr>
<tr>
<td>Ppp1r1a</td>
<td>82.5</td>
</tr>
<tr>
<td>Cort</td>
<td>89.0</td>
</tr>
<tr>
<td>GAPDH</td>
<td>87.2</td>
</tr>
<tr>
<td>B-actin</td>
<td>91.9</td>
</tr>
</tbody>
</table>
5 Discussion

Behaviour can be interpreted as the way in which an animal responds to stimuli, whether internal or external. However, the underlying molecular mechanisms controlling behaviour, especially complex behaviour, are still not well understood. A major problem in understanding the regulation of behaviour in complex organisms, is that there are many levels at which the nervous system can influence behaviour, from genes and transcripts, to cell-surface receptors, neural networks, circuits and competition between brain areas that converge on a behavioural output. Twin studies in humans have shown that regardless of identical DNA sequence and similar environments, variability in behaviour is present and sometimes quite strikingly different between co-twins. This suggests that contributors other than direct genetic or environmental influence may be responsible for differences in behaviour. Since behaviour can be modified by external as well as internal stimuli, attempting to discern part of the network of interactions responsible for producing individual idiosyncratic behaviour could help to explain, in part, complex behaviour. The goal of this thesis was to determine which candidate genes and their gene products contribute to measured behaviours, as measuring mRNA is an indirect way of assaying these extra-genetic components.

This thesis presents an experimental construct using multiple measures with three different analysis methods. Gene lists produced throughout the experiment have provided target genes for validation which have been previously implicated in developmental and psychiatric illnesses, which are capable of modifying the epigenome, of regulating transcription, of modulating or interacting with essential components of neurotransmission, which have been associated with variability in behaviour, the processes of neural differentiation, and in maintaining metabolic processes requisite for normal brain function, among others. The gene
products assayed using RT-PCR that did validate the arrays were of interest because they confirmed the array experiments. The gene products assayed using RT-PCR and which coordinated with behaviour indicated an association of that transcript with that particular behaviour. The following sections will outline the advantages and limitations of our study design.

5.1 Limitations

5.1.1 Experimental measures

Our study included testing behaviour across batches with a lengthy span of time separating experiments. Behavioural data for Experiment One was gathered in 2004; Experiment Two behavioural data were procured about a year later. This may have introduced genetic or epigenetic drift into the experimental data as a confounder. Additionally, the changing of some experimental conditions, such as using different activity monitoring boxes for locomotion and different elevated plus maze lighting between experiments, created the need to normalize the behavioural data for both experiments to be able to compare the batches at the behavioural and at the behaviour by gene expression levels.

For microarray data, since the two experiments were run at completely different time points on separate Affymetrix GeneChip® arrays, and because several different analysis methods were used based on the most current trends in array analysis, there is potentially an enormous amount of variability between the experiments based solely on these factors. This may have contributed to the difficulties of determining which transcripts in this system may be mediating a single given behaviour, especially when combined with selecting between two separate data sets generated from two separate behaviour measurement equipment as discussed above. As such,
analysis Method Two was implemented for final validation of Experiment Two arrays in an attempt to derive an overall picture for the entire project.

These technical and biological limitations may represent additional stringent measures applied to our Experiments, such as biologically determined stringency (i.e. determining the variability between batches in both behaviour and gene expression) which appears to have eliminated false positives from our analysis (see overlap of gene lists, Results section, Figure 13). The caveat to this is that these limitations may have produced some Type II error in our data, eliminating good candidates from gene lists and reducing probability of finding a genuine hit.

Complex behaviours are not generally the result of a single gene product and its influence on the whole animal. Often, hundreds of genes can contribute to these behaviours (Canli and Lesch, 2007), and subsequently many may contribute in subtle ways which are beyond the sensitivity of detection of currently available high-throughput platforms such as those used for this thesis.

5.1.2 Data quality and limitations

The overall quality of our arrays improved between Experiments One and Two, with reduced background noise and better signal and with a larger set of ProbeSets since we used the entire superset of the RAE230 GeneChip® set. However, there are certain limitations inherent in the use of microarrays with follow-up validation by RT-PCR.

For arrays, significant noise in the signal can be produced simply by processing tissue into mRNA to use as precursor for cDNA created for array hybridization. The assumption that the quantity of mRNA obtained from brain is equivalent between individual purification
reactions might lead to some skewing of results. This is because extraction of mRNA from brain
tissue is a multi-step process where the quantity and quality of the mRNA can be affected based
on very minor changes to the protocol. Since the tissue was processed in batches, it is likely that
some variation in extraction efficiency is present. However, the RNA used in our analysis was
of apparent good quality and by convention unlikely to affect data interpretation.

Limitations in data processing were directly proportional to the form of analysis
undertaken, i.e. correlative approaches (Method Two) vs. segregated approaches (SNR analysis).
One looked for strength of relationship between behaviour and array scores (Method Two), the
other for large differences between groups for which there may be no real large apparent
biological separation (SNR analysis). Since multiple gene products can work within a complex
interactome to generate a specific trait, making the assumption that genes with more expressed
mRNA have equivalent or greater in vivo efficacy or correlation in mediating behaviour is
perhaps a bit elementary. Second messenger systems are a good example of this phenomenon,
especially in systems where the primary message is limited in quantity but still drives extensive
downstream processes. For this reason, network analysis in future may be a better approach to
determining complex systems of function, as it may be able to link potential gene product
interactions instead of directing the researcher to evaluate individual genes.

5.1.3 Technical and biological limitations

Technical limitations to this study included variability in testing apparatus, changes in
animal handlers which has shown to affect behaviour (Hogg, 1996), development of
commercially available primer and assay systems with optimization to other tissues than brain,
tissue availability (small quantities), the potential for contamination of sample during dissection,
and the potential loss of mRNA during the extraction and purification process.
RT-PCR limitations were also present in our analysis. Technical difficulties with the ABI7000 being used during the first half of this thesis made all data acquired up to a certain point questionable in its accuracy, although a selected sampling from these data sets was conducted and showed equivalence to the initial data set. Although a selection of the same assays were run with the replacement ABI7500 fluorescence PCR cycler, to run all samples a second time would have severely limited our search using other gene expression assays due to limited mRNA availability. Primers for the given assays sometimes had quite poor efficiency, possibly due to low template concentration, or the potential for unknown splice variant interference with the assays. Optimization of each primer in brain tissue would have strained available biological and financial resources, but would improve future experiments conducted in this manner.

Biological limitations included some variability in the genome of the inbred strain of rats used. Unfortunately, Lewis rats, of all the rats selected for this genotyping project, have the greatest intra-strain variability, approximately of 20% of gene SNPs which were assayed by Smits et al (2004), based on the loci tested having more than one polymorphism. Because we attempted to verify the results of Experiment One by repeating the experiment with a larger number of animals, changing rat strains to a potentially less variable strain may have been counterproductive, or at best another very stringent biological control against false positives. This may have, regretably, increased also the number of false negatives by being too stringent in exclusion/inclusion criteria. Since our experiments were conducted to determine whether or not gene transcript number had an effect on behaviour, not necessarily how upstream regulation of transcript number affects downstream behaviour, this particular limitation may have skewed our results, or may explain in some ways the variability in behaviour seen within inbred strains. Determining genetic polymorphisms in known regulators and promoters of those genes as well
as in the genes themselves may provide some insight into phenotypic variability between inbred animal strains.

In addition to these constraints, additional limitations include the variability in several of the behaviours selected for study, including sugar feeding, EPM as previously mentioned, as well as locomotion measures, which may be related to circadian rhythms and feeding time preferences (Madrid et al., 1998), food texture and type preferences (i.e. sucrose pellet vs granulated sugar vs sugar dissolved in solution, vs chow preference), and individual responses of animals to handling stress (Strekalova et al, 2004). This lack of stability in many of the tests selected may have contributed significant noise to the transcriptional analysis. Repeated testing would have determined whether a more stable overall measure for these behaviours could be obtained, and allowed for further experimental control. The relative stability of PPI and startle measures suggests that these tests are less variable, but may also be explained by the fact that these tests involve many repeated trials. PPI and startle are inherently quick tests (taking only seconds for each trial) allowing many repeated measures, as compared to the other tests, which require a longer time, and thus less repetitions of the same measure. Additionally, it would have been prudent to rotate animals through the behaviour tests to control for test order effects, or to segregate animals into separate testing groups for each behaviour measured so as not to potentially confound expression patterns by exposing animals to stressful testing scenarios.

Tissue choice has a direct impact on the detection of transcriptional differences between and within individuals. Brain tissue is heterogeneous to begin with, and has been shown to have transcriptomic differences even to the level of individual neurons comprising morphologically similar regions of the CNS (Gong et al, 2003; Heintz et al, 2007 (Abstract)). This research suggests an unbelievable degree of complexity in neuronal and sub-neuronal organization within
regions of the brain, indicating that small differences in expression in particular neuronal subtypes may be eliminated during the indelicate process of tissue homogenization. Selecting brain regions more tightly connected with the behaviours assessed may have reduced transcriptomic noise in these experiments, and has been addressed in section 5.4 below.

The cell types occupying prefrontal cortex provide an admixed sample of mRNA from neuronal and non-neuronal cells such as glial cells, in addition to any contamination provided by taking fresh brain samples, such as the inclusion of RBCs, WBCs and their genetic material, among others. Small changes in gene expression responsible for a particular behaviour may be confined to a small subpopulation of cells (Heintz et al, 2007) that might be obscured by overall levels of transcription in the entire tissue sample, not to mention that PFC is a small volume of tissue often not exceeding 40mg in the adult rat.

5.1.4 Additional Limitations

Financial and technical constraints prevented an ideal experimental approach. The quantity of mRNA produced from experimental tissues was usually quite low, and barely surpassed the required quantities for both microarray and RT-PCR experimentation. The low quantities of extracted RNA may have been a result of brain tissue being high in fat, which interferes with the extraction process used. The poor yield and purity of mRNA extractions from brain have been noted previously in our laboratory in addition to others, and have been shown to be reduced compared to other tissue extractions of mRNA (Semeralul et al, 2005).

Microarrays limit our ability to infer downstream effects of transcription because they observe only transcript and not the functional protein levels that are the effectors of their gene products. This can be circumvented by conducting protein analysis but was, however, beyond the scope of this thesis. In addition to measuring protein expression levels, it is possible that post-
transcriptional modifications that may alter translation could have an effect on the weak assumption that mRNA translates to a relatively equal quantity of functional protein product.

There is also the possibility for experimental variation when using multi-phase processing for microarrays. To appropriately prepare data for analysis, there are at least 3 steps used for raw data pre-processing, with multiple algorithms available for each step, and used at the discretion of the statistician conducting the analysis, usually with some thought towards the experimental design and questions being asked of the array data. After array pre-processing, analysis becomes even more complex, with several steps including linear modeling with the number of different possible permutations of analytical method processing exceeding 100,000. The result of this huge potential for variability is the potential to generate completely different gene lists based entirely on statistical manipulation of the data, which may account for some of the lack of overlap between Experiment array data, or even within the same experiment using a different method of analysis.

Since there is no “gold standard” for array analysis, filtering, normalization or methods for significance testing for the large volume of data being generated using a microarray platform, methods depend on which platform was used as well as on the study objectives. Some technical variability within microarrays themselves results from possible cross-hybridization between highly homologous sequences on microarray platforms which could result in an incorrect ProbeSet assignment to a particular gene transcript.

Additionally, GO database annotation of ProbeSets can have certain limitations in the process of extracting biological meaning or functional motifs for downstream products of the transcripts. This is because the database is not comprehensive and is limited by the fact that it reflects only the currently available state of knowledge for the genes listed, which may not yet
describe their actual functions in tissues other than those described (Griffin et al, 2003, Zeeberg et al. 2003). Another technical challenge that using GO annotations can present is that since GO annotations are updated continually, searches for function or for clustering can change regularly.

The size of each gene list is sensitive to the selected p-value threshold used as a cut off. P-values selected for gene list creation as cutoffs correspond to statistically significant cutoffs correcting for false discovery rates, called the p-adjusted or q-value in this thesis. A p-value threshold is a potentially better way than generating a cutoff arbitrarily, i.e. based on a magnitude of differences between experimental comparisons such as fold change (Mobbs et al, 2004). A p-value of $10^{-4} = \text{one false positive for every 10,000 distinct pair-wise comparisons}$. The lower the p-value used as a threshold for significance, the more reliable the resulting gene list at the cost of false negatives. Cost-limitations in pursuing false positives generates the need to have a more stringent approach in microarray data significance thresholds.

5.2 Synopsis of Results

5.2.1 Behaviour

Behaviour scores show that the distribution of scores changed between Experiment One and Experiment Two (see Figure 8a), leading us to believe that either measurement of the available data between experiments needs a change in metric, or that some biologically relevant change in behaviour based on the environment, genetic or epigenetic drift, or that other stochastic changes in the gene-environment interactome is occurring. Regardless, the measures used to select array candidates from the animals tested remained relatively consistent across both Experiments in that the “high” group was significantly different in score than the “low” group for both measures. Because of these analyses, we can conclude that the degree of similarity of phenotype between individuals in a sample of animals from an inbred strain of rats, such as the
Lewis rats assayed in these experiments, is highly variable, allowing us to differentiate between stratified “high” and “low” performance categories.

Animals selected for expression profiling fell into either “high” or “low” categories and represented the extremes of the group of animals tested for behaviour, and were the groups selected for microarray analysis of gene transcription in the hopes that extremes of behaviour might also represent extremes, or at least significant differences, in gene transcription. The animals scores were correlated with the gene expression data, underwent linear modeling, and were produced into a gene list from which gene transcripts were selected for verification.

5.2.2 Gene Expression

Experiment One was initially validated using genes taken from the SNR analysis gene list. Candidate genes such as Hes1, Homer1 and Tieg/Klf10 showed strong correlations with behavioural scores and/or were also validated using RT-PCR. Hes1 was the best candidate for pursuit, resulting in an overall strong correlation between RT-PCR, behaviour, and array scores. These genes all appeared to be candidates for differences in a behavioural score, i.e. activity, between individuals from a single strain of rats. With these results, but with outstanding concerns about the power of the experiment, our laboratory chose to repeat it with a larger number of animals and a correspondingly greater number of arrays, in an attempt to replicate the gene list results of Experiment One.

The resulting gene list from analysis Method One of Experiment Two did not significantly overlap the gene list from Experiment One. This may have been due to analytical method discordance, as a repeated statistical test using Method Two eliminated this lack of congruency between the data sets for Experiment One and Experiment Two and there was much greater overlap in the subsequent gene lists produced from analysis with Method Two.
Regardless of this increased overlap, various gene transcripts were selected for assay purposes using RT-PCR.

Several of the selected genes are implicated in a spectrum of psychiatric disease disorders such as schizophrenia, bipolar disorder, and depression and include metabolic, structural, and developmentally linked genes, in addition to genes coding for neurotransmitter receptor subunits, chromatin remodeling or cellular trafficking proteins. The genes we selected for RT-PCR validation from Experiment Two, analysis Method One included Fbxl10, Nfib, Bbs2, Smarca4, and Snx27, for which only Smarca4 showed any array validation by RT-PCR. Some of these gene products correlated between RT-PCR and behaviour scores (see Table 4) of Locomotion and average startle, although the correlations were not consistent between separated “high” and “low” animals subjected to array analysis and the extended RT-PCR/behaviour score analysis that the other 30 rat samples were subjected to. This indicates that the behaviours being monitored, when subjected to molecular analysis, may represent only certain extreme aspects of behaviour, or may not resolve at all in such a small sample size (i.e. 10 rats sampled). The two most validated behavioural test measures were startle and total locomotion scores (Table 3a, Table 4). By reasoning, one might expect that behaviours resulting from or integrated into similar neurotransmitter tracts may share molecular or transcriptional similarities, more than behaviours which do not directly interface with the same pathways or which form from component portions of those tracts. Because of this, there may be greater correlation between gene lists derived from behavioural tests from similar neurotransmitter axes (i.e. dopaminergic; sugar feeding and activity behaviours) than resultant of behavioural tests which are not considered to be routed along the same pathways or projections (i.e. sugar feeding and EPM). However, the clear resolution of the transcriptional or molecular moderators of these behaviours by use of microarray technology may be obscured by individual neuronal transcriptional...
heterogeneity within a single tissue as described by the Gene Expression Nervous System Atlas (GENSAT) program.

However, the lack of validation for sugar feeding suggests that although sugar feeding and locomotion are thought to be mediated along the dopaminergic neurotransmitter tract, they seem to have very little in common in terms of transcriptional similarities in the PFC. The genes selected using analysis Method Two did not show validation with the arrays set from Experiment Two, though they did show some correlation with the startle behaviour (Table 4). This requires that the experimenters pose the question of “where is the disjoint between behaviour, array, and RT-PCR score?” The answer to this question is speculative at best: array data may not always coincide with RT-PCR data. This can happen if the primers for the PCR reactions are inefficient, if the RNA extracted does not accurately represent the RNA being expressed in living, functional brain, or if additional unknown splice variants of measured transcripts are being measured, or any additional disruption to the process of tissue processing right through array hybridization and PCR analysis which might compromise the entire assay’s sensitivity. Since our primers were from a commercially validated source (Applied Biosystems), and the calculated efficiencies appear to be acceptable, it is possible that the arrays simply do not represent the actual biology, or that the composition of RNA used to create cDNA for the purpose of RT-PCR validation does not represent the samples sent for array hybridization.

This last explanation is possible, as sample age towards the end of the validation protocol (by the time analysis Method Two was implemented) was >9 months, and the RNA had been freeze-thawed several times to generate cDNA to attempt to cross-validate Experiments. This may have reduced RNA quantity and quality from the original levels.
An additional consideration might be that the networks of molecules required to elicit complex behaviour in response to stimulus are not well identified, much less understood. It is possible that a single molecule may have profound downstream effects which, because of the nature of the cascade, may not appear to be important at the mRNA level. It could also be that many molecules work in conjunction to bring about the differential behaviour, at which point expression at the mRNA level may likely not accurately represent the functional interactions between these molecules, especially if they have variable life spans at the protein level.

5.3 Conclusions

Our experiments found that several mRNA transcripts correlated with behaviour in each of the two experiments, and in several cases the array results were validated by RT-PCR. However, there was little overlap between the two experiments. It is possible that there may be transcripts that consistently correlate with behaviour, for which DNA sequence determinants of transcription appear to be strong, allowing for a cohesive overlap of transcript expression to be seen between inbred strains of mice (Hovatta et al., 2005; unpublished data from our own laboratory). The lack of replication between our experiments suggests that there are other factors which are important in determining behaviour.

Our data then raises the question of which mechanism(s) mediate behavioural differences among inbred animals, since neither genes nor, as our data would suggest, transcript levels are the deterministic keystones. Possibly, behaviour is generated at a more macroscopic level, for example at the cell or even circuit level, for both of which detection is impossible using the approach taken in this thesis. The differences in transcript expression and behaviour could also be due to stochastic, environmental, or epigenetic contributions (Finch and Kirkwood, 2000), or an admix of all three in addition to circuit and molecular events, resulting in the inconsistencies
in transcript-behaviour association we observed, similar to previously made observations regarding lifespan within and between strains of mice (Gelman et al., 1988) and c.elegans (Brooks and Johnson, 1991).

5.4 Future Prospects

The design and power of this experiment does not appear to be sensitive enough to clearly associate gene expression with phenotype within an inbred strain. We have attempted to assess gene expression using a variation on this experimental design, using several different, well phenotyped strains of mice that show substantial variation across strains in a selected behaviour which is trait and not state-dependent, such as prepulse inhibition (Willott et al, 2003), and have had success in identifying and validating candidates which have shown variable levels in terms of expression in humans with schizophrenia who are more likely to have a deficit in prepulse inhibition of the acoustic startle response. Protein expression verification is the next step in this project.
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Appendix

Genes selected for RT-PCR validation of arrays for Experiment One and Experiment Two

**Abl-interactor 2 (Abi-2)**

The Abl-interactor proteins are linked to Abl and Src-family tyrosine kinases and are involved in cytoskeletal reorganization stimulated by growth factor. They are expressed both in mouse and in human, where Abi-2 is most highly expressed in the brain. Abl proteins from brain lysates have been shown to change in apparent molecular weight and phosphorylation state as the age of an animal increases. Abi-2 message is elevated both the central and peripheral nervous systems, but is expressed most highly in cortical layers receiving projected neurons. It is expressed both in embryonic and in post-natal brain, in post-natal and adult brain it has strongest expression in cerebral neocortex, hippocampus, and dentate gyrus (Courtney et al., 2000). Abi-2 encodes an SH3-containing protein with homology to DNA-binding proteins (Dai and Pendergast., 1995).

![Figure 17. Abi-2 Standard Curve.](image-url)
General transcription factor II I (GTF2I)

GTF2I encodes TFII-I, and is deleted in individuals with Williams-Beuren syndrome, a neurodevelopmental disorder which results in moderate mental retardation, personality and cognitive dysfunction (Wang et al., 1998) in addition to an overall reduction in brain volume, and changes in the size and shape of frontal and auditory cortex and cerebellum (Danoff et al., 2004). GTF2I encodes BAP-135, a protein which acts as a phosphorylation substrate of Bruton’s tyrosine kinase (Wang et al., 1998). GTF2I is present exclusively in neurons in the adult brain, with highest expression in hippocampal interneuron’s. Adult expression patterns show that the greatest expression of GTF2I mRNA is in hippocampus, neurons in the cerebral cortex and cerebellar Purkinje cells (Danoff et al., 2004).

Tropomodulin 2 (Tmod2)

Known as neuronal tropomodulin, this is a neuronal cytoskeleton protein which interacts with (Yang et al., 2006). It is predominantly expressed in the brain, and can be detected in rat brain as early as embryonic day 14 and reaches
adult expression levels before birth. It is specifically expressed in neurons from frontal cortex, and may associate with F-actin (Watakabe et al., 1996)

**Cryptochrome 2 (photolyase-like) (Cry2)**

Mammalian Cry2 is a blue-light photoreceptor requisite for maintaining circadian rhythms (van der Horst et al., 1999). It acts to generate negative feedback regulator in the autoregulatory transcriptional/translational feedback loops that maintain a circadian rhythm even with the absence of external time signals, and as such works as a circadian oscillator although the molecule itself is not light-responsive (Oster et al., 2003). Cry2 is important in the regulation of localized biological rhythms in all tissues (Oster et al., 2003).

**Cytochrome c oxidase subunit VIc-1 (Cox6c1)**

Cox6c is involved in the mitochondrial electron transport chain. Mitochondrial dysfunction in the brain has been implicated in

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Figure 20. Cry2 Standard Curve

![Cry2 Standard Curve](image)

\[ y = 4.0805x + 27.026 \]

\[ R^2 = 0.9870 \]

Figure 21. Cox6c1 Standard Curve

![Cox6c1 Standard Curve](image)

\[ y = 2.3564x + 34.753 \]

\[ R^2 = 0.7725 \]
various psychiatric disorders including bipolar disorder and schizophrenia, in more than one brain tissue (Sun et al., 2006).

**Down syndrome critical region gene 1-like 1 (Dscr1l1)**

Dscr1l1 belongs to a family of proteins that inhibit calcineurin activity, which can exert a variety of functions by dephosphorylating a family of transcription factors, and in the brain, affect synaptic plasticity, neuronal apoptosis, the regulation of ion channels as well as amyloid B-peptide formation. Expression is regulated by thyroid hormone (T3). Dscr1l1 alpha is the isoform (one of three) that is predominantly expressed in the cortex, and undifferentiated other regions in human brain. Beta isoforms are also expressed in the brain but are additionally expressed in non-neuronal tissues. Dscr1l1 expression is well preserved among mammalian species, although splice variants vary per species, and may have age-related variability in expression levels in the brain (Mizuno et al., 2004; Siddiq et al., 2001). This gene product has also been shown to regulate brain development and brain hypothyroidism has been shown to directly reduce expression of Dscr1l1 transcript production in rat brain, with transcript production increasing until P24 and then potentially plateauing in the adult rat, distributed most abundantly in hippocampus, dentate gyrus, and cerebral cortex neurons in layers I-VI (Siddiq et al., 2001).
High mobility group box transcription factor 1 (Hbp1)

Hbp1 is a non-histone chromosomal protein which acts as a transcription factor and is widely expressed throughout the adult rat brain and is localized to the nuclei of almost all neurons as well as oligodendrocyte-like cells. (Kim et al., 2008). The gene is induced in post-ischemic rat brain and may be a therapeutic target for stroke (Takata et al., 2004).

Syntaxin 3 (Stx3)

Syntaxin 3 is produced in most cell types, including epithelial cells, and interacts with vesicle release machinery to bring about exocytosis at least at the apical plasma membrane in the gut (Galli et al., 1998) from the trans-golgi network (ter Beest et al., 2005). It is widely expressed throughout the human body, but in rat brain syntaxin 3 has a distinctive pattern of expression, being highest in striatum and cerebellum (Chen et al., 1999). It may also play a role in tonic release of neurotransmitter in the retina since it has been correlated with ribbon synapses (Morgans et al., 1996).
Regulating synaptic membrane exocytosis 1 (Rims1)

Rims1 transcript generates a brain-specific protein product that binds Rab3a-binding protein (Sun et al., 2003) and participates in the synaptic vesicle cycle and are located in active zones of conventional synapses (Betz et al., 2001). Rims1 product interacts directly with Ca\textsuperscript{2+} channels, synaptotagmin, and SNAP-25, and thought to control docking and fusion of secretory vesicles at the plasma membrane (Coppola et al., 2001). It also confers neurotransmitter vesicle anchoring in addition to sustained activity to presynaptic voltage dependent Ca\textsuperscript{2+} channels (Kiyonaka et al., 2007). Also interesting, the N-terminal domain of Rim1 protein has been shown to engage in a high-affinity interaction with 14-3-3 (Sun et al., 2003).

Hairy and enhancer of split 1 (Hes1)

Hes1 gene expression is required for maintenance of neural progenitors in embryonic brain although it can inhibit proliferation and differentiation if expression levels are persistently high. Hes1 gene product oscillations regulate downstream gene

Figure 25. Rims1 Standard Curve.

Figure 26. Hes1 Standard Curve.

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transcription which in turn maintains neural progenitors by Notch signaling activation (Shimojo et al., 2008). Hes1 expression is essential for neural stem cell pool maintenance, inhibiting neuronal differentiation (Bai et al., 2007) and regulating the development of the central nervous system including both cranial and spinal nerve systems (Hatakeyama et al., 2006).

**Homer, neuronal immediate early gene, 1 (Homer1)**

Homer proteins are implicated in learning, memory, and addiction and are involved in the functional assembly of postsynaptic proteins at glutamatergic synapses. Homer1 knockout mice have behavioural and neurochemical abnormalities that coincide with those modeled in animals, including impaired prepulse inhibition, anxiety, reactivity to novel environments, reduced sucrose intake, enhanced behavioural despair, and deficits in memory tests such as radial arm maze (Szumlinski et al., 2005). Two separate isoforms, constitutively expressed and immediate early gene, have distinct functional differences in the PFC in the mediation of behaviour, and in the maintenance of basal extracellular glutamate and regulation of PFC glutamate release (Lominac et al., 2005).
Glutamate receptor, ionotropic, N-methyl D-aspartate-1 (Grin1)

Grin1 encodes the NMDA receptor NR1 subunit and some of its markers have been positively associated with schizophrenia, the best SNP to date indicating this relationship being rs11146020 (Georgi et al., 2007; Zhao et al., 2006). The NR1 subunit is the binding site for glycine and D-serine (both potent activators acting as neurotransmitters) in the NMDA receptor. The NR1 subunit may play a role in memory-related signal transduction mediated by magnesium block of the channel. NMDA receptors may also mediate neuroinflammation, apoptosis, and may be responsible for excitotoxicity (Lang et al., 2007).

TGFB inducible early growth response/ Krupple like factor 10 (Tieg/Klf10)

Tieg encodes a three zinc-finger Kruppel-like transcription factor which has a role in cellular differentiation, apoptosis, proliferation and migration by inhibiting growth. It does so by binding to type I and type II serine/threonine kinase receptors which signal internally by Smad proteins, which

Figure 28. Grin1 Standard Curve.

Figure 29. Tieg/Klf10 Standard Curve.
appear to be the primary mediators of Tieg signal transduction. Altered Tieg expression has been noted in several varied cancers (Johnsen et al., 2002). In brain which has been damaged, Tieg is induced early after the damaging incident and is expressed at >1.3 fold from undamaged brain for at least 24hr after injury (Kobori et al., 2002). Chronic treatment with fluoxetine has been shown to increase Tieg expression in prefrontal cortex, hippocampus and in the dentate gyrus (Alme et al., 2007)

**Neurexin 3 (Nrxn3)**

Neurexins are a superfamily of cell adhesion molecules that work as presynaptic neuroligin cell surface receptors. They also act as receptors for neurexophilins, dystroglycans, neuroligins and $\alpha$-latrotoxins. Nrxn3 appears to have several SNPs that are associated with alcohol dependence (Hishimoto et al., 2007) and has an $\alpha$ and a $\beta$ isoform, like all other neurexins, each of which have subtly different neuronal and synaptic functions (Ushkaryov and Sudhof, 1993). Nrxn3 clustering can indicate the recruitment of synaptic vesicles to synaptic terminals and recruitment of synaptic machinery (Dean et al., 2003).
Phosphatidylinositol binding clathrin assembly protein (Picalm)

Picalm (also known as CALM) has been shown to mediate the assembly of clathrin and is involved in coated-vesicle formation, and important step in intracellular trafficking of membrane compartment components. It is expressed ubiquitously throughout the body, and interacts with SH3 domains, indicating an interaction with Src protein (Kim and Kim, 2001).

F-box and leucine-rich repeat protein 10 (Fbxl10)

Fbxl10 acts as a histone H3 demethylase. It complexes with Ring1B/Rnf2, a histone H2A monoubiquitilation enzyme which is associated with transcriptional repression (Sanchez et al., 2007), repressing the transcription of ribosomal RNA genes and has been identified as a growth regulator and as a putative tumor suppressor. It preferentially binds the transcribed region of ribosomal DNA to
repress its transcription. Fbxl10 is expressed in low levels in aggressive brain tumors, suggesting that the combination of these two mechanisms of transcriptional regulation may contribute to cellular transformation and cancer development (Frescas et al., 2007).

**Nuclear factor 1 B-type (Nfib)**

Nfib is a transcription factor gene which is essential for brain development, and lacking the gene product, mice have late gestation neuroanatomical defects such as agenesis of the corpus callosum, reductions in the volume other forebrain commissures and the loss of specific midline glial populations. It may also be responsible for the development of hippocampus and pons during late gestational stages, and is certainly responsible for the retardation of lung development during gestation, resulting in pseudo-viable animals with attenuated lifespans and with death at birth of Nfib–null animals (Steele-Perkins et al., 2005).

**Bardet-Biedl syndrome 2 protein (BBS2)**

Part of a family of genes (Bbs) which confer, through inheritance of three alleles, Bardet-Biedl Syndrome (Katsanis et al., 2001)
which is a pleiotropic genetic disorder which features obesity, photoreceptor density, developmental delay, and polydactyly among others. The gene is expressed early in development and continues to be expressed in many adult tissues (Sheffield, 2004). Amino acid sequence is highly conserved in mammals, and disruption of this sequence in individuals with Bardet-Biedl Syndrome may contribute to the obesity aspect of the phenotype (Laurier et al., 2006), however its current functional properties are as of yet unknown.

**SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a, member 4 (Smarca4)**

Smarca4’s gene product is an ATP-dependent chromatin remodeling complex which is highly conserved in eukaryotes. It plays a role in cell cycle regulation, and can act as a tumor suppressor. It alters the translational position of nucleosomes to make DNA accessible to factor binding, creating access to binding sites near entry and exit point on a nucleosome (Fan et al., 2005). Smarca4 protein product has a role in cell growth control and differentiation, and may regulate the transcription of 5-6% of the genome in yeast (Medina et al., 2005)

![Figure 35. Smarca4 Standard Curve.](image)
Sorting nexin 27 (Snx27)

Snx27 is a cellular trafficking protein which contains a phox-homology domain, which is a lipid- and protein-interaction domain found in NADPH oxidase complex in phagocytes, in addition to a RasGTP effector domain which stimulates the dissociation of GDP from Ras-related proteins, allowing GTP to bind and activate GTPases (Worby and Dixon, 2002). There are two transcript variants (Snx27a and Snx27b). Both protein isoforms have been shown to interact with 5-HT4aRs by targeting them to early endosomes (Joubert et al., 2004).

Protein phosphatase 1 regulatory subunit 1A (Ppp1r1a)

This protein phosphatase inhibitor interacts with second messenger system camp, regulating other downstream proteins and processes. It is inactivated by calcineurin, and by phosphatase regulation mediates synaptic plasticity. PPP1R1A has 85% peptide identity between human and rat brain tissue, and is a
functional homolog of DARPP-32 (PPP1R1B) (Endo et al. 1996), which has been implicated in the mediation of multiple neuronal signals and in several psychiatric diseases.

**Cortistatin precursor (Cort)**

Cort produces a precursor of a secreted neuropeptide (preprocorticostatin) which is structurally similar to somatostatin. Its mRNA is expressed in a distinct subset of interneuron’s in rat cerebral cortex and hippocampus in regions important to high cognitive functions (de Lecea et al., 1997). Despite the structural similarity to somatostatin, and the fact that it binds all somatostatin receptors with near analogous affinity, there appear to be receptors that will selectively bind corticostatin. Corticostatin binds ghrelin/growth hormone secretagogue receptor and appears to modulate growth hormone release in a dose-dependent fashion (Luque et al., 2006) and has been shown to regulate sleep patterns, and has been demonstrated to be neuroprotective against kainite-induced neurotoxicity (Spier and de Lecea, 2000).

![Cort Standard Curve](image)

Figure 38. Cort Standard Curve
**Gamma-aminobutyric acid receptor subunit gamma-1 (Gabrg1)**

Gabrg1 encodes the gamma-1 subunit of the GABA(A) receptor and has been associated with alcohol dependence in concordance with Gabrg2 variant expression (Ittiwut et al., 2008). GABRG1 has been implicated in autistic-spectrum disorder through interaction with other GABRG subunits (Ma et al., 2005).

**Cyclin-dependent kinase 5 (Cdk5)**

Cdk5 is a neuronal protein kinase which may play a role in abnormally phosphorylating tau proteins, a major component of accumulative peptide in Alzheimer’s (Poon et al., 1997). It is predominantly expressed in postmitotic neurons (Wang et al., 2006). It is essential for neurite outgrowth during neuronal differentiation, neuronal migration, and the laminar configuration of the cerebral cortex during development, modulation of glutamatergic and DAergic...
transmission, endocytosis of synaptic vesicles (Wang et al., 2006), its kinase activity increases
during neurogenesis when complexed with p35, independent of other cyclin. CDK complexes
(Poon et al., 1997), and it may play a role in neuroprotection (Wang et al., 2006).