Published in final edited form as:

Alcohol Clin Exp Res. 2007 July ; 31(7): 1089–1098. doi:10.1111/j.1530-0277.2007.00397.x.

Identification of Candidate Genes for Alcohol Preference by Expression Profiling of Congenic Rat Strains

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Abstract

Background—A highly significant quantitative trait locus (QTL) on chromosome 4 that influenced alcohol preference was identified by analyzing crosses between the iP and iNP rats. Congenic strains in which the iP chromosome 4 QTL interval was transferred to the iNP (NP.P) exhibited the expected increase in alcohol consumption compared with the iNP background strain. This study was undertaken to identify genes in the chromosome 4 QTL interval that might contribute to the differences in alcohol consumption between the alcohol-naïve congenic and background strains.

Methods—RNA from 5 brain regions from each of 6 NP.P and 6 iNP rats was labeled and analyzed separately on an Affymetrix Rat Genome 230 2.0 microarray to look for both *cis*-regulated and *trans*-regulated genes. Expression levels were normalized using robust multi-chip average (RMA). Differential gene expression was validated using quantitative real-time polymerase chain reaction. Five individual brain regions (nucleus accumbens, frontal cortex, amygdala, hippocampus, and striatum) were analyzed to detect differential expression of genes within the introgressed QTL interval, as well as genes outside that region. To increase the power to detect differentially expressed genes, combined analyses (averaging data from the 5 discrete brain regions of each animal) were also carried out.

Results—Analyses within individual brain regions that focused on genes within the QTL interval detected differential expression in all 5 brain regions; a total of 35 genes were detected in at least 1 region, ranging from 6 genes in the nucleus accumbens to 22 in the frontal cortex. Analysis of the whole genome detected very few differentially expressed genes outside the QTL. Combined analysis across brain regions was more powerful. Analysis focused on the genes within the QTL interval confirmed 19 of the genes detected in individual regions and detected 15 additional genes. Whole genome analysis detected 1 differentially expressed gene outside the interval.

Conclusions—*Cis*-regulated candidate genes for alcohol consumption were identified using microarray profiling of gene expression differences in congenic animals carrying a QTL for alcohol preference.

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Keywords

Alcohol Consumption; QTL; Microarray; Candidate Genes; Congenic Rats

Genes Contributing to alcoholism are difficult to identify due to both the genetic complexity of alcoholism and environmental variability among alcoholic subjects. While various techniques can be utilized to identify possible molecular and physiological patterns associated with alcoholism, localizing the primary genetic determinants for the risk to develop alcoholism has proven to be difficult. Nevertheless, several genes have been associated with a risk for alcoholism in studies that have been replicated, e.g. *GABRA2* (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005), *ADH4* (Edenberg et al., 2006; Guindalini et al., 2005; Luo et al., 2005a), and *CHRM2* (Luo et al., 2005b; Wang et al., 2004). It is evident, however, that there are other genes that also influence the risk for alcoholism. Selective breeding of rodents for alcohol-related traits, followed by quantitative trait locus (QTL) analysis has located genomic regions in which variation affects the phenotype. However, finding the specific genes within such regions is difficult. A strategy of making congenic animal models carrying a single QTL, and then using microarray analysis to determine which genes are altered in expression, should provide a powerful approach toward gene identification (Hitzemann et al., 2004; Hoffman and Tabakoff, 2005; Spence et al., 2005). Using this combined approach dramatically decreases the number of genes that are expected to be differentially expressed because the 2 strains are identical except for the QTL sequences, thereby increasing the probability to identify differentially expressed genes contributing to a specific phenotype such as alcohol preference.

The alcohol-preferring (P) and alcohol-nonpreferring (NP) rat lines were developed, through bidirectional selective breeding, from a randomly bred, closed colony of Wistar rats, on the basis of alcohol preference in a 2-bottle choice paradigm (Li et al., 1991). Alcohol-preferring rats display the phenotypic characteristics considered necessary for an animal model of alcoholism (Cicero, 1979; Li et al., 1993). Subsequently, inbred P (iP) and NP (iNP) strains were established; these inbred strains maintain highly divergent alcohol consumption scores (Carr et al., 1998). Owing to the physiological and genetic similarity between humans and rats, iP and iNP rats can be studied to identify important genetic factors that might influence alcoholic predisposition in humans.

A highly significant QTL that influenced alcohol preference was identified on chromosome 4 (with a maximum lod score of 9.2) in a cross between iP and iNP rats; suggestive QTLs were identified on chromosomes 3 and 8 (Bice et al., 1998; Carr et al., 1998). The chromosome 4 QTL acts in an additive fashion and accounts for approximately 11% of the phenotypic variability. Owing to the strong association between the chromosome 4 QTL region and alcohol preference, this approximately 25 cM region is likely to harbor at least 1 gene that directly contributes to alcohol preference. Reciprocal congenic strains in which the iP chromosome 4 QTL interval was transferred to the iNP (NP.P) and the iNP chromosome 4 QTL interval was transferred to the iP (P.NP) exhibited the expected effect on alcohol consumption of the strain that donated the chromosome 4 QTL interval (Carr et al., 2006).

This indicates that the chromosome 4 QTL region is, in part, responsible for the disparate alcohol consumption observed between the iP and iNP rats.

The objective of the present study was to identify genes in the chromosome 4 QTL interval that might be responsible for the divergent alcohol preference of the iNP and iP strains. Because the chromosome 4 QTL interval is the only region that differs between the 2 strains, analyzing only the probe sets in this interval focuses on the *cis*-regulated genes, and increases power by reducing the number of comparisons. Analysis of all the probe sets allows detection of both *cis*-regulated and *trans*-regulated genes, but at the expense of testing more probe sets [and therefore a higher false discovery rate (FDR) and lower power]. Discrete brain regions were analyzed for expression differences between the homozygous NP.P and the iNP background strain, to detect differences that might be localized to 1 region. Expression data from the 5 brain regions of each animal were then combined to obtain an average expression level for analysis of genes that may differ across multiple regions; combining the 5 regions in a single animal reduces technical variation and should increase power to detect differences that are in the same direction but that may fall below significance in individual regions. These complementary analyses should allow us to better understand the inherent differences in gene expression between strains that differ in alcohol preference as a result of genetic differences in a single chromosomal region.

MATERIALS AND METHODS

Animals

Creation of the NP.P congenic strain has been described previously (Carr et al., 2006). Briefly, the NP.P congenic strain was initiated by crossing 1 male rat from the iP5 strain with 1 female rat from the iNP1 strain to create iPiNP F1 animals, which were backcrossed to the iNP1 strain to produce the N2 generation. The presence of the chromosome 4 interval was confirmed using microsatellite markers. Ten generations of backcrossing to the iNP1 strain were performed, followed by an intercross between N10 animals to produce homozygous animals (N10F1), which resulted in the finished congenic strain.

All animals were individually housed on a standard 12 hour:12 hour light:dark cycle and were provided nutritionally balanced rat chow and water ad libitum. Animals were habituated (by the same experimenter) to handling and to the guillotine daily between 09:00 and 10:00 hours for 10 days before being killed. The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the Institutional Animal Care and Use Committee and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, NIH, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

A total of 12 (6 iNP and 6 NP.P) male rats, 14 to 15 weeks of age, were killed by decapitation between 09:00 and 10:00 hours over 2 consecutive days, with equal numbers of animals from each strain killed each day. This minimized differences in time of killing and

dissection, and maintained the experimental balance across the 2 strains. The head was immediately immersed in chilled isopentane (−50 °C) for 15 seconds and then placed in a cold box maintained at −15 °C, where the brain was rapidly removed and placed on a glass plate for dissection. All equipment used to obtain tissue was treated with RNaseZap (Ambion Inc., Austin, TX) to prevent RNA degradation. The nucleus accumbens, caudate putamen, frontal cortex, hippocampus, and amygdala were dissected as described previously (Liang et al., 2004).

RNA Isolation

Dissected tissues were immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's protocol, but with triple the suggested ratio of Trizol to tissue (Edenberg et al., 2005). Ethanol-precipitated RNA was further purified through RNeasy® midi columns (Qiagen, Valencia, CA), according to the manufacturer's protocol. To avoid DNA contamination in the real-time polymerase chain reaction (PCR) assay, the RNA was treated with 1 U of DNase I for 20 minutes at 37 °C. Total RNA yields in each region did not differ between the congenics and the iNP animals ($p>0.47$); the average yields in the different regions ranged from 69 to 97 μg. The quality of the RNA from all rats and regions was similar, as monitored by absorbance spectra from 210 to 350 nm, by electrophoresis on 1% agarose gels, and using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm the ribosomal bands.

RNA Labeling and Microarray Hybridization

RNA from each individual rat was labeled and hybridized separately on an Affymetrix Rat Genome 230 2.0 microarray. Starting with 5 μg of total RNA from each animal, first and second-strand cDNA synthesis was carried out according to the standard protocols from Affymetrix (Affymetrix: *GeneChip® Expression Analysis Technical Manual*, Santa Clara, CA, Affymetrix, 2001). Biotinylated cRNA was synthesized in vitro from the double-stranded cDNA using the ENZO BioArray High Yield RNA Transcript Labeling Kit (ENZO Diagnostics, Farmingdale, NY) according to the Affymetrix protocol. Fifteen micrograms of fragmented, biotinylated cRNA was mixed into 300 μL of hybridization cocktail, of which 200 μL was used for each hybridization. Hybridization was carried out for 17 hours at 42 °C. Washing, staining, and scanning were carried out according to the standard protocol.

To minimize potential systematic errors, all stages of the experiment were balanced across phenotypes. That is, equal numbers of NP.P and iNP animals were killed each day, and equal numbers of RNA preparations from iP and iNP animals were processed through the labeling, hybridization, washing, and scanning protocols on each day, in different alternating orders. Whenever possible, common premixes of reagents were used to minimize effects due to differences in reagent preparation.

Data Analysis and Informatics

Each GeneChip® was scanned using an Affymetrix Model 3000 scanner and underwent image analysis using Affymetrix GCOS software. Microarray data are available from the National Center for Biotechnology Information's Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/ (Barrett et al., 2005; Edgar et al., 2002) under series Accession

No. GSE5849 (GSM136446 … GSM136505). Raw .cel files were imported into the statistical programming environment R (R Development Core Team, 2006) for further analysis with tools available from the Bioconductor Project (Gentleman et al., 2004). Expression data were normalized and $\log_2$ transformed using the robust multichip average (RMA) method (Bolstad et al., 2003; Irizarry et al., 2003) implemented in the Bioconductor package RMA.

Mapping of probe sets to chromosomal locations was accomplished with data provided by Affymetrix. The identities of the probe sets were confirmed by comparing the target mRNA sequences on the Affymetrix Rat Genome 230 2.0 GeneChip with the NCBI nucleotide databases. Any probe set that could not be annotated was culled from the list, leaving 28,049 probe sets for further analysis. To increase power and decrease the FDR, probe sets were subjected to further filtering to remove those probe sets not reliably detected by the arrays (McClintick and Edenberg, 2006). Using the detection call generated by the Affymetrix Microarray Analysis Suite 5.0 algorithm, probe sets were retained that had a fraction present of at least 0.33 in at least 1 group of samples in the comparison (McClintick and Edenberg, 2006). For the analyses by brain region, the probe sets were retained if probe sets had a fraction present ≥ 0.33 for either the congenic NP.P or iNP animals for that brain region (20,300–21,800 probe sets for individual regions). For the analyses by average expression level, probe sets were used that had a fraction present ≥ 0.33 for at least 1 brain region in at least 1 strain (23,079 probe sets).

Genewise *p* values were calculated by *t*-tests performed using the package Limma (Smyth, 2004). False discovery rate was calculated by the method of Benjamini and Hochberg (1995). Probe sets were considered to be differentially expressed if the FDR was <0.10. First, each individual brain region was analyzed separately. Then, data from the 5 brain regions from each animal were averaged and the average value was analyzed to detect differences that were more general across multiple regions. To focus on *cis*-regulated genes, probe sets that mapped to the chromosome 4 QTL region between 29,413,686 and 128,186,835 base pairs (4q13–4q34), locations of the microsatellite markers *D4Rat151* and *D4Rat55* that flanked the introgressed region, were analyzed separately (960 probe sets).

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was carried out using SYBR Green chemistry and the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). The amplification primers were designed from the sequence in the coding region of the gene using Vector NTI (Invitrogen); when possible, at least 1 primer spanned an exon/intron boundary. Total RNA, isolated for the microarray analyses, was used for these analyses. Following reverse transcription of the RNA [Superscript III reverse transcription system for RT-PCR (Invitrogen)], an aliquot of each reverse transcription reaction was amplified in triplicate. The qRT-PCR was repeated to generate 6 values for each test sample. Two control reactions were run for each RNA preparation: (1) a reverse transcription and PCR reaction with no added RNA to control for contamination of the reagents and (2) a PCR reaction without the reverse transcription reaction to detect DNA contamination of the RNA preparation. To correct for sample-to-sample variation, an endogenous control (GAPDH)

was amplified with the target and served as an internal reference to normalize the data. The average GAPDH C_t values for NP.P and iNP were the same in each brain region tested, making this an appropriate control gene to normalize the expression of the candidate genes of interest. Relative quantification of data from the ABI Prism 7700 sequence detection system was performed using the standard curve method (Applied Biosystems, User Bulletin #2; http://www.appliedbiosystems.com).

RESULTS

Cis-Regulated Genes

Microarray analyses were performed to detect differences between the congenic NP.P and inbred NP (iNP) rats in gene expression in 5 brain regions (nucleus accumbens, frontal cortex, amygdala, hippocampus, and striatum). These regions were selected based on their inclusion in the mesolimbic and mesocortical systems, both of which are critically important in the initiation and maintenance of goal-directed and reward-mediated behaviors (reviewed in Bonci et al., 2003 and Maldonado, 2003). Because the only genetic differences between these 2 strains are in the QTL region on chromosome 4, examining just the set of genes located in this region allows the most powerful test of *cis*-regulated candidate genes. More differences (at FDR <0.10) were detected in the frontal cortex, amygdala, and striatum, and the fewest differences in the nucleus accumbens (Table 1). Three genes differed in all 5 brain regions; these are not well annotated, but have some homology to known genes. Ppm1k_predicted [protein phosphatase 1 K (PP2C domain containing) (predicted)] was lower (about 2-fold) in all 5 regions in NP.P (Table 1). LOC685879 (similar to juxtaposed with another zinc finger protein 1) was higher (about 1.6-fold) in NP.P. RGD1304879 [similar to zinc finger protein 398 (binding protein p52/p71)] was higher (about 1.3-fold) in NP.P. Two genes, Snx10 (sorting nexin 10, involved in intracellular trafficking) and Tsga13 [testis-specific gene A13 (predicted)] differed in 4 of the 5 regions, and were 1.5-fold higher and about 1.3-fold lower in NP.P, respectively. Two genes (Akr1b10 and Nfe213) each differed in 3 regions and another 10 differed in 2 of the regions. The direction of the differences is similar when comparing the NP.P and iNP.

To detect more widespread differences in gene expression, data from the 5 discrete brain regions of each animal were averaged. This reduces random technical variation from the individual extractions and labeling, and thereby provides more power to detect differences that are in the same direction in multiple regions but may fall below significance in individual regions. In the analysis focused on the chromosome 4 QTL region, 35 genes within the region differed between the 2 strains; 12 were lower in NP.P and the remaining 23 were higher in NP.P (Table 2). Most of the differences were small. Neuropeptide Y (Npy) and alpha synuclein (Snca), previously identified candidate genes for alcohol consumption in the iP and iNP rats (Liang et al., 2003; Spence et al., 2005), differed in expression in this combined analysis.

Trans-Regulated Genes

To detect *trans*-regulated genes (genes identical in the 2 strains that are differentially expressed due to variations in a regulatory gene located within the chromosome 4 region),

we analyzed all of the probe sets on the array. This reduces the power to detect differences because the very large amount of additional testing increases the FDR. As would be expected when comparing the congenic strain with the background strain, nearly all of the probe sets that exhibited significant differences in expression between the NP.P and iNP were located in the chromosome 4 QTL interval. Some of the genes that were significant in the analysis of the chromosome 4 region alone no longer met the criteria of FDR <0.10. At an FDR <0.10, 6 differences were detected in amygdala, 8 in striatum, and 4 in the frontal cortex; none were detected in either nucleus accumbens or the hippocampus (Table 1). Only 4 *trans*-regulated genes were detected, 3 in the amygdala and 1 in the frontal cortex, and all were expressed at lower levels in the NP.P strain (Table 1).

In the combined analysis of the 5 brain regions using all probe sets, nearly all of the differences (34/35) were in genes located in the chromosome 4 region (Table 2). Some of the genes detected in our analysis of chromosome 4 probe sets alone no longer met FDR <0.10 when the larger number of comparisons was performed. The one *trans*-regulated gene that was detected, Sos1 (Son of sevenless homolog 1), is important in signal transduction.

The mapped locations of the transcripts and their direction of expression are shown in Fig. 1. We noticed that several clusters of adjacent genes were coordinately up-regulated or down-regulated in the congenic animals. For example, except for RGD1306697, 9 transcripts that mapped between Plxna4 and RGD130487 were expressed more in the NP.P animals. Seven of the next 8 transcripts were expressed at lower levels in the NP.P.

Confirmation by qRT-PCR

To confirm genes that differed in expression between the NP.P and iNP, qRT-PCR was performed. Based on the literature reports of their possible involvement in pathways related to alcohol-seeking behavior, 9 genes were selected for confirmation. The first gene selected for confirmation was diacylglycerol kinase, iota (*Dgki*); all 5 brain regions were tested and the direction of expression was in the expected direction in all 5 regions. The remaining genes were tested in the striatum (Table 3). The differential expression of *Akr1b4*, *Grid2*, *Baiap1*, *Dgki*, *Snca*, and *Ppm1k* was confirmed at $p<0.005$ (Table 3). The expression of *Sos1* and *Npy* was in the expected direction.

DISCUSSION

The NP.P strain, with the iP Chr 4 QTL interval introgressed onto the iNP background, exhibits the expected increase in alcohol consumption compared with the iNP littermates (Carr et al., 2006). By examining gene expression in this congenic strain, we limited the differences expected to those originating from genetic variation within the QTL region shown to affect the phenotype of alcohol preference. This allowed us to identify candidate quantitative trait genes (QTGs) for the alcohol preference phenotype. Because the 2 strains are identical except for a region on chromosome 4, the a priori expectation is that only *cis*-regulated genes located in that region of chromosome 4 or genes *trans*-regulated by genes within that region should differ. This is expected to be a small set of genes, the signal from which could be masked by random variations in the very large set of genes that do not differ. The genes within the QTL interval that differ in expression are by definition *cis*-regulated

and thus QTG candidates. By focusing on the region that differed, and thereby limiting the number of comparisons and increasing the power of the analysis, we were able to detect differences in the expression of *cis*-regulated genes.

Analysis of all of the probe sets allows us to detect *trans*-regulated genes, but at the expense of reduced power due to the much larger number of comparisons and therefore higher FDR. As expected, nearly all of the genes that differed in expression in the analysis of all probe sets were located in the chromosome 4 region that differed, and are candidates for *cis*-acting QTGs.

The number of genes that differed in each individual region was not large. Many of the genes were differentially expressed (in the same direction) in more than 1 region; 3 (Ppm1k, LOC685879, and RGD1304879) in all 5 regions, 2 in 4 regions, and 2 in 3 regions. By combining the regions in a joint analysis, we were able to detect more differences (compare Tables 1 and 2). Many genes are expected to be expressed under similar regulatory control in different brain regions. This combined analysis has more power to detect genes that are changed in the same direction in multiple regions but might not meet the strict FDR within individual regions, because averaging the 5 individual measurements from each animal that contribute to the comparison reduces technical variation. When focusing on the genes within the QTL interval, 19 of the genes detected in individual regions were confirmed in the combined analysis and 15 additional genes were detected.

Nine genes were tested by qRT-PCR to confirm their differential expression; expression of 8 genes was in the expected direction, with 7 being significant at $p<0.005$. The exponential nature of the q-PCR reactions makes them less able to detect small differences in expression. The primers for these confirmation studies, when possible, were in the coding sequences spanning an intron. It has been our experience that when primers are designed based on the coding region, as we did here, the number of confirmed genes is lower than when using primers designed within the 3′ sequences used on the microarray chips. The overall level of confirmation is comparable to the FDR we had set (<0.1).

The grouping of transcripts that were expressed higher or lower was evident when observed in their mapped positions (Fig. 1). One can speculate that these regions of up-regulation and down-regulation are due to transcriptional control by an epigenetic effect, such as methylation patterns and histone modification. Covalent modifications of histone proteins, RNA-associated gene silencing processes, and DNA methylation are components of the epigenetic mechanism controlling gene expression patterns. These modifications have the potential to work together to modify the spatial structure of the DNA and the chromatin associated with it, to establish structural states that are either favorable (open chromatin) or not favorable (closed chromatin) for gene expression (Callinan and Feinberg, 2006). Future experiments could examine epigenetic regulation.

Programs to classify genes into overrepresented categories are not powerful when dealing with small numbers of differentially expressed genes, as in this QTL study. Thus, for the purpose of the discussion, 5 categories (cell signaling, protein trafficking, synaptic function, metabolism, and gene expression) were selected based on the ontology of the differentially

expressed genes in the chromosome 4 QTL region. In these categories, we discussed potential candidate genes that were detected in multiple brain regions and/or with multiple probe sets with at least a 15 to 20% difference in expression. We also discuss genes that have previously been implicated in alcohol preference.

Candidate genes involved in cell signaling include *Dgki*, BAI-associated protein 1 (*Baiap1*), and protein phosphatase type 1 K (*Ppm1k*). DGKs are a family of enzymes that regulate the levels of various pools of diacylglycerol (DAG), affecting DAG-mediated signal transduction. Dgki catalyzes the phosphorylation of DAG, an activator of protein kinase C, to phosphatidic acid (PA) and thus down-regulates second messenger pathways activated by protein kinase C (Van Blitterswijk and Houssa, 1999), which play important roles in regulating behavioral responses to ethanol (Newton and Messing, 2006). The highest expression of Dgki, when studied in humans, is found in the brain (Ding et al., 1998); we found that it was expressed at higher levels in the NP.P brain regions compared with the iNP brain regions. This is similar to findings in the alcohol-accepting (AA) and alcohol-nonaccepting (ANA) animals selectively bred for alcohol preference from Wistar stock, where *Dgki* is expressed at higher levels in discrete brain regions of the AA compared with the ANA animals (Sommer et al., 2001).

Baiap1 is a novel member of the membrane-associated guanylate kinase (MAGUK) homolog family and an isoform of the neurone-specific synaptic scaffolding molecule (S-SCAM), which is known to interact with NMDA and delta 2 glutamate receptors (*Grid2*) (Dobrosotskaya et al., 1997); *Grid2* was expressed at lower levels in the frontal cortex, amygdala, and combined analysis presented here (Tables 1 and 2). Membrane-associated guanylate kinase proteins participate in the assembly of multiprotein complexes on the inner surface of the plasma membrane at regions of cell–cell contact. Membrane-associated guanylate kinase proteins share a common modular structure that consists of multiple PDZ domains and a C-terminal guanylate kinase domain (Fanning and Anderson, 1999). They appear to have both structural and signaling roles within the cell and aid in localizing and coupling components of specific signal transduction pathways at specific subcellular locations. Another PDZ domain protein, multiple PDZ domain protein (*Mpdz*), has been identified as a QTG for alcohol and pentobarbital withdrawal seizures in mice (Shirley et al., 2004).

Ppm1k encodes a serine/threonine protein phosphatase. Together with protein kinases, these enzymes control the state of phosphorylation of cell proteins and thereby provide an important mechanism for regulating cellular activity.

Candidate genes involved in protein trafficking include Plexin A4 (*Plxna4*), sorting nexin 10 (*Snx10*), and src family-associated phosphoprotein 2 (*Scap2*). Plxna4 mediates multiple semaphorin signals, which are important in the development of the nervous system (Suto et al., 2005), and plays roles in a wide variety of axon guidance events in a highly regulated manner (Ben-Zvi et al., 2006). The protein Scap2 is an src-kinase associated protein. It plays a role in regulating the phosphorylation of Snca (Takahashi et al., 2003), a previously identified candidate gene for alcohol consumption in the iP/iNP animals.

The candidate gene, glutamate receptor ionotropic delta 2 (Grid2), is involved in signal transduction and synaptic function. The *Grid2* gene encodes the ionotropic glutamate receptor channel delta-2 subunit, functionally related to the amino-methyl-isoxazoleproprionate (AMPA) receptor (Landsend et al., 1997). Mutated *Grid2* has an increased permeability to glutamate in *Xenopus* oocytes (Taverna et al., 2000) and to calcium in HEK 293 cells (Wollmuth et al., 2000). *Grid2* is located within a common fragile site (CFS), which is a large region of genomic instability found in all individuals (Smith et al., 2006), making it an especially likely target for mutations involving genetic rearrangement (Robinson et al., 2005). Many of the large CFS genes, greater than 99% intronic, are involved in neurological development. Whether there are instability-induced alterations in the iP/iNP *Grid2* gene that influence its expression and in turn play a role in alcohol preference will require further investigation.

Candidate genes involved in metabolism include aldoketo reductase 1, member B4 (*Akr1b4*), and *Akr1b10*. Aldo-keto reductases catalyze the reduction of aliphatic and aromatic aldehydes to their corresponding alcohols.

Candidate genes involved in gene expression include zinc finger protein 398 and juxtaposed with another zinc finger protein 1. Many zinc finger proteins bind nucleic acids and regulate transcription.

Two previously identified candidate genes for alcohol preference, *Npy* and *Snca*, differed in expression when the data from the 5 discrete brain regions of each animal were averaged (Table 2). *Npy* expression was lower in the congenics than in the iNP animals; it was previously shown to be lower in iP than in iNP (Spence et al., 2005). The expression of *Snca* was lower in the congenic animals compared with the iNP animals (Table 2), whereas previous studies of the iP versus iNP showed higher expression in the iP animals (Liang et al., 2003), suggesting that Snca may be more prominently regulated by *trans*-regulatory than *cis*-regulatory factors.

The changes that were detected were small: only 10 of the 35 genes differed more than 20% (Table 2). Other comparisons of brain expression have also reported small differences. In a study that identified differentially expressed genes between the AA and ANA rat lines, the majority of differences reported were less than 50% (Worst et al., 2005). Likewise, most of the differences in expression levels in the hippocampi of iP and iNP alcohol-naïve rats were less than 50% (Edenberg et al., 2004). In another study, brain gene expression differences between the C57BL/6 and DBA/2 mice within QTL were compared; again, most of the differences were small (Hitzemann et al., 2004).

In summary, very interesting candidate genes and possible pathways have been identified by expression profiling of the congenic NP.P strain. The alteration in expression of *Dgki* (down-regulates PKC), *Grid2* (phosphorylated by PKC), *Scap2* (inhibits phosphorylation), and *Ppm1k* (serine/threonine phosphatase) strongly suggests that signal transduction pathways, especially the PKC pathway, are involved in the development of alcohol preference in the iP rats. Thus, the findings from this study provide interesting candidate genes for future functional and knockout studies, and information to other investigators who

are using these animals to study weight, anxiety (personal communication), bone mass, and bone strength (Alam et al., 2005).

Acknowledgments

This work was supported by the National Institute on Alcohol Abuse and Alcoholism Grants AA10707 and AA07611. The microarray studies were carried out using the facilities of the Center for Medical Genomics at Indiana University School of Medicine. The Center for Medical Genomics is supported in part by the Indiana Genomics Initiative of Indiana University, INGEN, which is supported in part by the Lilly Endowment Inc.

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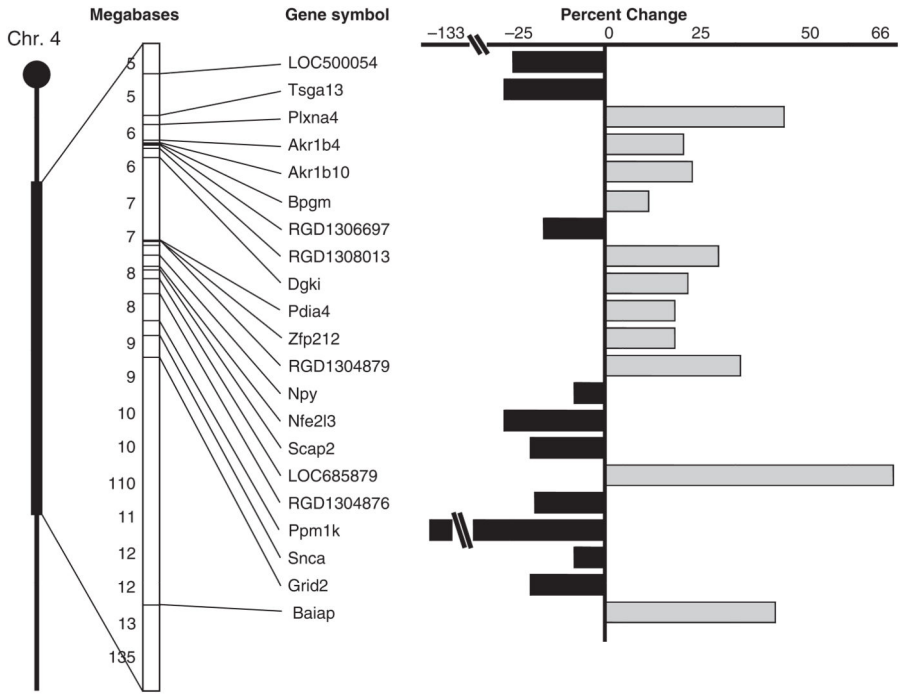

Fig. 1.

Mapping of the genes differentially expressed between the NP.P congenic strain and the iNP strain. The thick line on the left designates the Chr 4 quantitative trait locus region. Gene symbols and their physical locations (megabases) are on the left and the percent difference and direction are on the right.

Table 1

Differentially Expressed Genes in Individual Brain Regions

Probeset	UniGene	Gene symbol	Gene name	Location	Fold change	*p*-value	FDR-chr4	FDR-all
Amygdala								
1394939_at	Rn.117902	**Ppm1k**	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.67	0.0000	0.0000	0.0000
1378742_at	Rn.161004	**LOC685879**	Similar to juxtaposed with another zinc finger protein 1	4q24	1.64	0.0000	0.0001	0.0031
1379275_at	Rn.44192	**Snx10**	Sorting nexin 10	4q24	1.40	0.0000	0.0007	0.0232
1390625_at	Rn.40681	**RGD1304879**	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.40	0.0000	0.0021	0.0521
1383828_at	Rn.59832	**Tsga13**	Testis-specific gene A13 (predicted)	4q22	− 1.32	0.0000	0.0026	0.0587
1368778_at	Rn.9968	**Slc6a6**	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	4q34	− 1.48	0.0001	0.0054	0.0977
1384146_at	Rn.18604	Luc7l2	LUC7-like 2 (*S. cerevisiae*) (predicted)	4q22	− 1.31	0.0003	0.0133	0.1656
1397959_at	Rn.8058	RGD1310722	Similar to RIKEN cDNA D130059P03 gene (predicted)	4q22	− 1.45	0.0008	0.0349	0.2244
1372525_at	Rn.12713	Fkbp14	FK506-binding protein 14	4q24	1.19	0.0010	0.0400	0.2371
1382409_at	Rn.162740	Tsga14	Testis specific, 14	4q22	− 1.12	0.0016	0.0480	0.2683
1390042_at	Rn.162736	MGC109491	Similar to 1110007F12Rik protein	4q22	1.32	0.0015	0.0480	0.2683
1370007_at	Rn.39305	Pdia4	Protein disulfide isomerase-associated 4	4q24	1.24	0.0017	0.0511	0.2785
1378543_at	Rn.162746	Nfe2l3	Nuclear factor, erythroid derived 2, like 3 (predicted)	4q24	− 1.23	0.0023	0.0618	0.2955
1387482_at	Rn.10046	Grid2	Glutamate receptor, ionotropic, delta 2	4q31	− 1.21	0.0044	0.0937	0.3668
1398553_at	Rn.16505	Tmed5	Transmembrane emp24 protein transport domain containing 5	14p22	− 1.40	0.0000	NA	0.0587
1375320_at	Rn.18688	RGD1311019	Similar to hypothetical protein DKFZp434H2010 (predicted)	5q36	− 1.28	0.0000	NA	0.0761
1376299_at	Rn.134218	LOC312678	Similar to Retinoblastoma-binding protein 2 (RBBP-2)	4q42	− 1.35	0.0001	NA	0.0872
Frontal cortex								
1394939_at	Rn.117902	**Ppm1k**	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.57	0.0000	0.0000	0.0002
1378742_at	Rn.161004	**LOC685879**	Similar to juxtaposed with another zinc finger protein 1	4q24	1.71	0.0000	0.0000	0.0003
1367734_at	Rn.107801	**Akr1b4**	Aldo-keto reductase family 1, member B4 (aldose reductase)	4q22	1.29	0.0000	0.0002	0.0066
1379275_at	Rn.44192	**Snx10**	Sorting nexin 10	4q24	1.58	0.0000	0.0002	0.0066
1379480_at	Rn.52190	LOC500082	Similar to diacylglycerol kinase iota	4q22	1.24	0.0002	0.0096	0.1756
1383828_at	Rn.59832	Tsga13	Testis-specific gene A13 (predicted)	4q22	− 1.26	0.0002	0.0096	0.1756
1378543_at	Rn.162746	Nfe2l3	Nuclear factor, erythroid derived 2, like 3 (predicted)	4q24	− 1.35	0.0004	0.0152	0.2192
1383551_at	Rn.108143	Bpgm*	2,3-bisphosphoglycerate mutase	4q22	1.16	0.0004	0.0155	0.2339
1370902_at	Rn.23676	Akr1b8	Aldo-keto reductase family 1, member B8	4q22	1.16	0.0005	0.0158	0.2465

Probeset	UniGene	Gene symbol	Gene name	Location	Fold change	*p*-value	FDR-chr4	FDR-all
1392541_at	Rn.129060	RGD1304876	Similar to RIKEN cDNA A030007L17; EST AA673177 (predicted)///similar to RIKEN cDNA A030007L17 (predicted)	4q24	− 1.26	0.0010	0.0291	0.3545
1390625_at	Rn.40681	RGD1304879	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.20	0.0011	0.0313	0.3677
1395190_at	Rn.8475	Akr1b10	Aldo-keto reductase family 1, member B10 (aldose reductase)	4q22	1.21	0.0014	0.0363	0.3813
1386777_at	Rn.138054	LOC500054	Similar to POT1-like telomere end-binding protein	4q22	− 1.21	0.0017	0.0436	0.4081
1393607_at	Rn.10046	Grid2	Glutamate receptor, ionotropic, delta 2	4q31	− 1.14	0.0022	0.0499	0.4148
1368778_at	Rn.9968	Slc6a6	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	4q34	− 1.35	0.0021	0.0499	0.4101
1389291_at	Rn.106389	Chchd3	Coiled-coil-helix-coiled-coil-helix domain containing 3 (predicted)	4q22	− 1.15	0.0041	0.0748	0.4807
1387786_at	Rn.3239	Mtpn	Myotrophin	4q22	− 1.14	0.0039	0.0748	0.4765
1373746_at	Rn.158722	RGD1306697	Similar to HSPC049 protein (predicted)	4q22	− 1.13	0.0040	0.0748	0.4800
1370007_at	Rn.39305	Pdia4	Protein disulfide isomerase-associated 4	4q24	1.19	0.0048	0.0850	0.4854
1368297_at	Rn.34322	Gata2	GATA-binding protein 2	4q34	1.15	0.0057	0.0945	0.5053
1394361_a_at	Rn.134460	Wnt2	Wingless-related MMTV integration site 2	4q21	1.16	0.0058	0.0945	0.5074
1371239_s_at	Rn.37575	Tpm3	Tropomyosin 3, γ	2q34	− 1.26	0.0000	NA	0.0609
Striatum								
1394939_at	Rn.117902	**Ppm1k**	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.05	0.0000	0.0000	0.0000
1395190_at	Rn.8475	**Akr1b10**	Aldo-keto reductase family 1, member B10 (aldose reductase)	4q22	1.38	0.0000	0.0001	0.0026
1379275_at	Rn.44192	**Snx10**	Sorting nexin 10	4q24	1.55	0.0000	0.0001	0.0026
1379480_at	Rn.52190	**LOC500082**	Similar to diacylglycerol kinase iota	4q22	1.27	0.0000	0.0001	0.0041
1378742_at	Rn.161004	**LOC685879**	Similar to juxtaposed with another zinc finger protein 1	4q24	1.43	0.0000	0.0008	0.0264
1367734_at	Rn.107801	**Akr1b4**	Aldo-keto reductase family 1, member B4 (aldose reductase)	4q22	1.24	0.0000	0.0009	0.0286
1378543_at	Rn.162746	**Nfe2l3**	Nuclear factor, erythroid derived 2, like 3 (predicted)	4q24	− 1.25	0.0000	0.0020	0.0654
1373746_at	Rn.158722	**RGD1306697**	Similar to HSPC049 protein (predicted)	4q22	− 1.23	0.0001	0.0029	0.0927
1380094_a_at	Rn.152206	Zfp212	Zinc finger protein 212	4q24	1.16	0.0003	0.0090	0.2725
1390625_at	Rn.40681	RGD1304879	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.22	0.0003	0.0113	0.3284
1394973_at	Rn.10289	Pde1c	Phosphodiesterase 1C	4q24	1.16	0.0004	0.0123	0.3594
1392541_at	Rn.129060	RGD1304876	Similar to RIKEN cDNA A030007L17; EST AA673177 (predicted)///similar to RIKEN cDNA A030007L17 (predicted)	4q24	− 1.26	0.0006	0.0167	0.4490
1394585_at	Rn.7020	RGD1562563	Similar to RIKEN cDNA G430041M01 (predicted)	4q24	− 1.45	0.0011	0.0291	0.5520
1389291_at	Rn.106389	Chchd3	Coiled-coil-helix-coiled-coil-helix domain containing 3 (predicted)	4q22	− 1.16	0.0035	0.0797	0.7663
Nucleus accumbens								
1378742_at	Rn.161004	LOC685879	Similar to juxtaposed with another zinc finger protein 1	4q24	1.80	0.0000	0.0131	0.4197

Probeset	UniGene	Gene symbol	Gene name	Location	Fold change	*p*-value	FDR-chr4	FDR-all
1390625_at	Rn.40681	RGD1304879	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.39	0.0000	0.0131	0.4197
1395190_at	Rn.8475	Akr1b10	Aldo-keto reductase family 1, member B10 (aldose reductase)	4q22	1.27	0.0001	0.0183	0.4727
1383828_at	Rn.59832	Tsga13	Testis-specific gene A13 (predicted)	4q22	− 1.25	0.0008	0.0636	0.9213
1392510_at	Rn.23539	RGD1308013	Similar to hypothetical protein B230314O19 (predicted)	4q22	1.43	0.0010	0.0744	0.9213
1394939_at	Rn.117902	Ppm1k	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.36	0.0005	0.0454	0.9213
Hippocampus								
1378742_at	Rn.161004	LOC685879	Similar to juxtaposed with another zinc finger protein 1	4q24	1.76	0.0000	0.0034	0.1097
1394939_at	Rn.117902	Ppm1k	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.05	0.0001	0.0069	0.2236
1390625_at	Rn.40681	RGD1304879	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.36	0.0001	0.0069	0.2236
1399134_at	Rn.138054	LOC500054*	Similar to POT1-like telomere end-binding protein	4q22	− 1.22	0.0001	0.0080	0.2591
1379275_at	Rn.44192	Snx10	Sorting nexin 10	4q24	1.58	0.0008	0.0454	0.9758
1390042_at	Rn.162736	MGC109491	Similar to 1110007F12Rik protein	4q22	1.14	0.0013	0.0600	0.9758
1383828_at	Rn.59832	Tsga13	Testis-specific gene A13 (predicted)	4q22	− 1.21	0.0014	0.0600	0.9758
1397419_at	Rn.49269	Mpp6	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) (predicted)	4q24	1.22	0.0023	0.0825	0.9758

* Genes significant in more than one probe set.

Differences between NP.P and iNP in each of 5 brain regions were analyzed individually. Fold change is the ratio of the expression level of NP.P to iNP; a negative number indicates the ratio of expression of iNP to NP.P. FDR-chr4 is the false discovery rate from the analysis of probe sets in the chromosome 4 region, FDR-all is the value for analysis of all probe sets. The criterion for transcripts to be significantly differentially expressed was FDR<0.1. The bold gene symbols represent the transcripts significant when all probe sets were used for analysis.

Table 2

Differentially Expressed Genes in Combined Analysis of All Regions

Probeset	UniGene	Gene symbol	Gene name	Location	Fold change	*p*-value	FDR-chr4	FDR-all
1378742_at	Rn.161004	**LOC685879**	Similar to juxtaposed with another zinc finger protein 1	4q24	1.66	0.0000	0.0000	0.0000
1394939_at	Rn.117902	**Ppm1k**	Protein phosphatase 1K (PP2C domain containing) (predicted)	4q24	− 2.33	0.0000	0.0000	0.0000
1390625_at	Rn.40681	**RGD1304879**	Similar to zinc finger protein 398 (zinc finger DNA-binding protein p52/p71)	4q24	1.31	0.0000	0.0000	0.0000
1395190_at	Rn.8475	**Akr1b10**	Aldo-keto reductase family 1, member B10 (aldose reductase)	4q22	1.20	0.0000	0.0000	0.0000
1378956_at	Rn.169275	**Plxna4***	Plexin A4 (predicted)	4q22	1.41	0.0000	0.0000	0.0001
1367734_at	Rn.107801	**Akr1b4**	Aldo-keto reductase family 1, member B4 (aldose reductase)	4q22	1.18	0.0000	0.0000	0.0001
1383828_at	Rn.59832	**Tsga13**	Testis-specific gene A13 (predicted)	4q22	− 1.23	0.0000	0.0000	0.0001
1377198_at	Rn.162747	**Scap2***	src family-associated phosphoprotein 2	4q24	− 1.17	0.0000	0.0080	0.0003
1381871_at	Rn.154502	**Baiap1**	BAI1-associated protein 1 (predicted)	4q34	1.39	0.0000	0.0001	0.0005
1378543_at	Rn.162746	**Nfe2l3**	Nuclear factor, erythroid derived 2, like 3 (predicted)	4q24	− 1.23	0.0000	0.0000	0.0013
1379480_at	Rn.52190	**Dgki**	Similar to diacylglycerol kinase iota-1	4q22	1.19	0.0000	0.0001	0.0014
1392510_at	Rn.23539	**RGD1308013**	Similar to hypothetical protein B230314O19 (predicted)	4q22	1.26	0.0000	0.0001	0.0028
1389710_at	Rn.91844	**Sos1**	Son of sevenless homolog 1 (Drosophila)	6q11	1.15	0.0000	NA	0.0028
1380094_a_at	Rn.152206	**Zfp212**	Zinc finger protein 212	4q24	1.16	0.0000	0.0002	0.0046
1373746_at	Rn.158722	**RGD1306697**	Similar to HSPC049 protein (predicted)	4q22	− 1.14	0.0000	0.0004	0.0106
1387482_at	Rn.10046	**Grid2***	Glutamate receptor, ionotropic, delta 2	4q31	− 1.17	0.0000	0.0006	0.0158
1388544_at	Rn.108143	**Bpgm**	2,3-bisphosphoglycerate mutase	4q22	1.10	0.0000	0.0010	0.0294
1370007_at	Rn.39305	**Pdia4**	Protein disulfide isomerase-associated 4	4q24	1.16	0.0000	0.0012	0.0337
1392541_at	Rn.129060	**RGD1304876**	Similar to RIKEN cDNA A030007L17; EST AA673177 (predicted)///similar to RIKEN cDNA A030007L17 (predicted)	4q24	− 1.16	0.0001	0.0015	0.0427
1386777_at	Rn.138054	**LOC500054***	Similar to POT1-like telomere end-binding protein	4q22	− 1.21	0.0001	0.0020	0.0586
1389291_at	Rn.106389	Chchd3	Coiled-coil-helix-coiled-coil-helix domain containing 3 (predicted)	4q22	− 1.13	0.0002	0.0040	0.1150
1390042_at	Rn.162736	MGC109491	Similar to 1110007F12Rik protein	4q22	1.18	0.0005	0.0094	0.2409
1379275_at	Rn.44192	Snx10*	Sorting nexin 10	4q24	1.39	0.0006	0.0101	0.2535
1367977_at	Rn.1827	Snca	Synuclein, alpha	4q24	− 1.07	0.0009	0.0158	0.3316
1376481_at	Rn.162758	Adamts9	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9 (predicted)	4q34	1.18	0.0011	0.0179	0.3544
1376576_at	Rn.24649	Dusp11	Dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	4q34	1.08	0.0020	0.0311	0.4978

Probeset	UniGene	Gene symbol	Gene name	Location	Fold change	*p*-value	FDR-chr4	FDR-all
1397419_at	Rn.49269	Mpp6*	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) (predicted)	4q24	1.11	0.0025	0.0373	0.5468
1373190_at	Rn.134425	Cnot4	CCR4-NOT transcription complex, subunit 4	4q22	1.08	0.0027	0.0397	0.5503
1387154_at	Rn.9714	Npy	Neuropeptide Y	4q24	− 1.07	0.0028	0.0401	0.5526
1375836_at	Rn.117708	RGD1561018	Similar to hypothetical protein (predicted)	4q22	1.06	0.0033	0.0466	0.6043
1380105_at	Rn.16045	RGD1565367	Similar to Solute carrier family 23, member 2 (Sodium-dependent vitamin C transporter 2) (predicted)	4q22	1.08	0.0035	0.0473	0.6043
1377600_at	Rn.20401	RGD1566056	Similar to KIAA1285 protein (predicted)	4q24	1.08	0.0035	0.0473	0.6043
1387954_a_at	Rn.15680	Grip2	Glutamate receptor interacting protein 2	4q34	1.08	0.0073	0.0824	0.7208
1397692_at	Rn.50685	Tia1	Cytotoxic granule-associated RNA-binding protein 1	4q34	1.09	0.0077	0.0857	0.7376
1397153_at	Rn.7990	Lmbrd1	LMBR1 domain containing 1	4q34	− 1.08	0.0088	0.0959	0.7823

*Genes significant in more than one probe set.

Differences between NP.P and iNP when the 5 discrete brain regions of each animal were combined. The bold gene symbols represent the transcripts that were significant when all probe sets were used for analysis. Other details are the same as those in Table 1.

Table 3

qRT-PCR Confirmation of Microarray Data

	Fold change[a]	
Gene name	**Microarray**	**qRT-PCR**
Akr1b4	1.18	1.28[b]
Baiap	1.39	1.67[b]
Dgki	1.19	2.19[b]
Ppm1K	− 2.33	– 1.83[b]
Snca	− 1.07	– 1.48[b]
Grid2	− 1.17	– 1.64[b]
Sos1	1.15	1.05
Npy	− 1.07	− 1.04
Zfp212	1.16	– 1.82[c]

[a] Fold change is the ratio of the expression level of NP.P to iNP; a negative number indicates the ratio of expression of iNP to NP.P.

[b] Fold change for qRT-PCR is significant at $p<0.005$.

[c] Fold change is significant, but not in the expected direction.