Differences in Peptidylglycine α-amidating Monooxygenase in C57 and DBA mice.

Rebecca Ganetzly
Bioinformatics and Bioengineering Summer Institute, 2004
Laboratory of Dr. Michael Miles.

Abstract

The inbred strains of mice: C57BL/6J (C57) and DBA/2J (DBA) show different ethanol-drinking behaviors. Namely, C57 mice are less sensitive to, but will voluntary consume more than DBA mice. Affymetrix microarray data suggest that peptidylglycine α-amidating monooxygenase (PAM) is more highly expressed in DBA mice than in C57 mice. Natural language processing literature searching programs, and quantitative trait locus analysis have also suggested possible roles for PAM in ethanol drinking behavior. However, while the microarray results have been confirmed with PCR, the genomic DNA for the three-prime untranslated region (UTR) of PAM is approximately 50 nucleotides longer in C57 mice. Investigation into the performance of individual Affymetrix probes for PAM suggests that probes are being affected by this short difference of sequence between the strains of mice.

Introduction

The addiction to alcohol is a devastating disorder that is increasingly thought to have a genetic component. An individual who has a genetic predilection towards becoming an alcoholic will behave differently, even on his or her first exposure to alcohol than one without a predisposition. Namely, someone who has a genetic tendency towards alcoholism will be less sensitive to the physiological and behavioral effects of alcohol. An inbred strain of mice, C57BL/6J, is a genetic model for an alcoholic – it shows less effect from ethanol consumption and chooses to consume more ethanol than other mice. By contrast, DBA/2J mice show a high sensitivity to ethanol and do not voluntarily consume ethanol (1). To explain why these mice have different behaviors when given ethanol, we can look to the genes being expressed by the different mice, both with and without ethanol treatment. Using Affymetrix microarrays to compare the basal level of gene expression between these two mice, we find a high basal difference in peptidylglycine α-amidating monooxygenase (Michael Miles, unpublished data). Therefore, PAM is a candidate gene in causing the different ethanol-preference behaviors in these different mice, and potentially is a target gene for preventing alcoholism in humans. For this microarray data to be viewed as meaningful, however, it must be verified by a method with higher reliability, and relevancy of PAM to alcoholism must be established.

Microarray data can be highly unreliable, as Affymetrix's 20mer probes are sensitive to single nucleotide polymorphisms between experimental DNA and the source DNA used to fabricate the probe (Michael Miles, unpublished data). Also, PAM has

been shown in rats to have a total number of seven splice variants, of which two do not contain the complementary sequence to that used in the microarray (2-4). Another cause for concern in the veracity of the microarray results is that PAM-2, although it contains the 3' UTR exon from which the Affymetrix probes were made, was reported to have a version of this exon that was 20 nucleotides shorter than PAM-1, even though no splicing has been reported in this region (4).

Simply because PAM has a basal difference in expression level between C57 and DBA mice does not mean that PAM is connected to ethanol drinking behavior. In order for the differential expression of PAM to be relevant, there should be other indications that PAM is connected to Ethanol behavior.

Methods

Bioinformatics Methods – determining a method for PAM's effects

In examining possible connections to ethanol, a plethora of bioinformatics tools were used. WebQTL's correlation tool was used to find the top 500 genes whose expression correlated with PAM's using both the Pearson's and Spearman's methods (5). This correlation was repeated for each of the three principal components WebQTL generated for PAM. WebQTL's cluster tree function was also used on all of the Perfect Match (PM) probes of PAM, to show QTLs mapped against the mouse chromosomes.'

Affymetrix microarray data was analyzed using the provided MAS 4 software. Significance of differences was analyzed using the S-score and position dependent nearest neighbor methods (6,7). Genes that did not have an average difference of at least 50 in one of the 18 experiments were filtered out (three biological replicates were used for each treatment – ethanol or saline. For each mouse a separate microarray experiment was run with mRNA from the prefrontal cortex, nucleus accumbens and ventral tegmental area) were filtered out. A SAM multiclassed response analysis using 300 permutations and k-nearest neighbor inputer was conducted, and the number of significant genes were chosen so that the false significance prediction approximated 10%. S-scores were then averaged across biological replicates and correlated across all brain regions and treatments with PAM using both Spearman and Pearson methods. Genes with a correlation coefficient below .9 were filtered out. This analysis was done with Affymetrix MU74Av2, MU74Bv2 and MU74Cv2 chips.

Bibliosphere, a literature searching tool that uses natural language processing to find literature connections was used to create a putative list of related genes to peptidylglycine α-amidating monooxygenase.

The correlation lists of genes generated from WebQTL and microarray analysis and the gene list generated by Bibliosphere were submitted as Affymetrix probe IDs to EASEonline (9). LocusLink Mouse was used as the background gene list.

The lists generated by WebQTL, microarray analysis and bibliosphere were submitted in sequential sets of 50 to Chilibot, another literature searching tool, along with the term "alcohol" and synonyms "alcoholic," "alcoholism," and "ethanol." In addition to Chilibot's provided synonyms, the synonyms "α-amidation," "α-amidate," "α-amidating," "PAL," and "PHM" were used for PAM.

Exploring PAM splicing in mice

To investigate the possibilities of splice variants in mice, the rat sequence (as provided by NCBI) and mouse sequence (as provided by the UCSC genome browser) for each exon of PAM were aligned using NCBI BLAST two sequence alignment.

MotifFinder was also used to search the 3' UTR for the consensus sequence for polyadenylation, which we would expect to find if there were a splice variant of PAM that terminated in the middle of the 3' UTR.

Biological confirmation of PAM expression differences

Nothern blotting was done from a 1.5% agarose gel, made with 722 mL DEPC-treated water, 17.8 mL of formaldehyde at 65º and 10 mL of 10x MOPS buffer at 65º. Total RNA was loaded from DBA and C57, saline and ethanol-treated mice from the prefrontal cortex, and ventral tegmental area, at 5.0 μg, speedvacuumed to be under 3.0 μls. Total RNA from the nucleus accumbens at 4.0 μg. Riboprobes were created from PCR products ligated to T7 promotors, using the Lig'n scribe kit. Riboprobe creation was done with the E-Z strip kit. The blot was first probed with a sequence from PAM exons 3 and 4, then stripped using the E-Z strip kit, and probed with a sequence from the Affymetrix target sequence. Primer pairs were ATGCCTTGGTACCACCAGAC (5') and GCAGCATATGGTGGACAGTG (3'); and TCAGCCTGTGCCAGTAAAGA (5') and AAGACATCCCGGAAACACAC(3'), respectively.

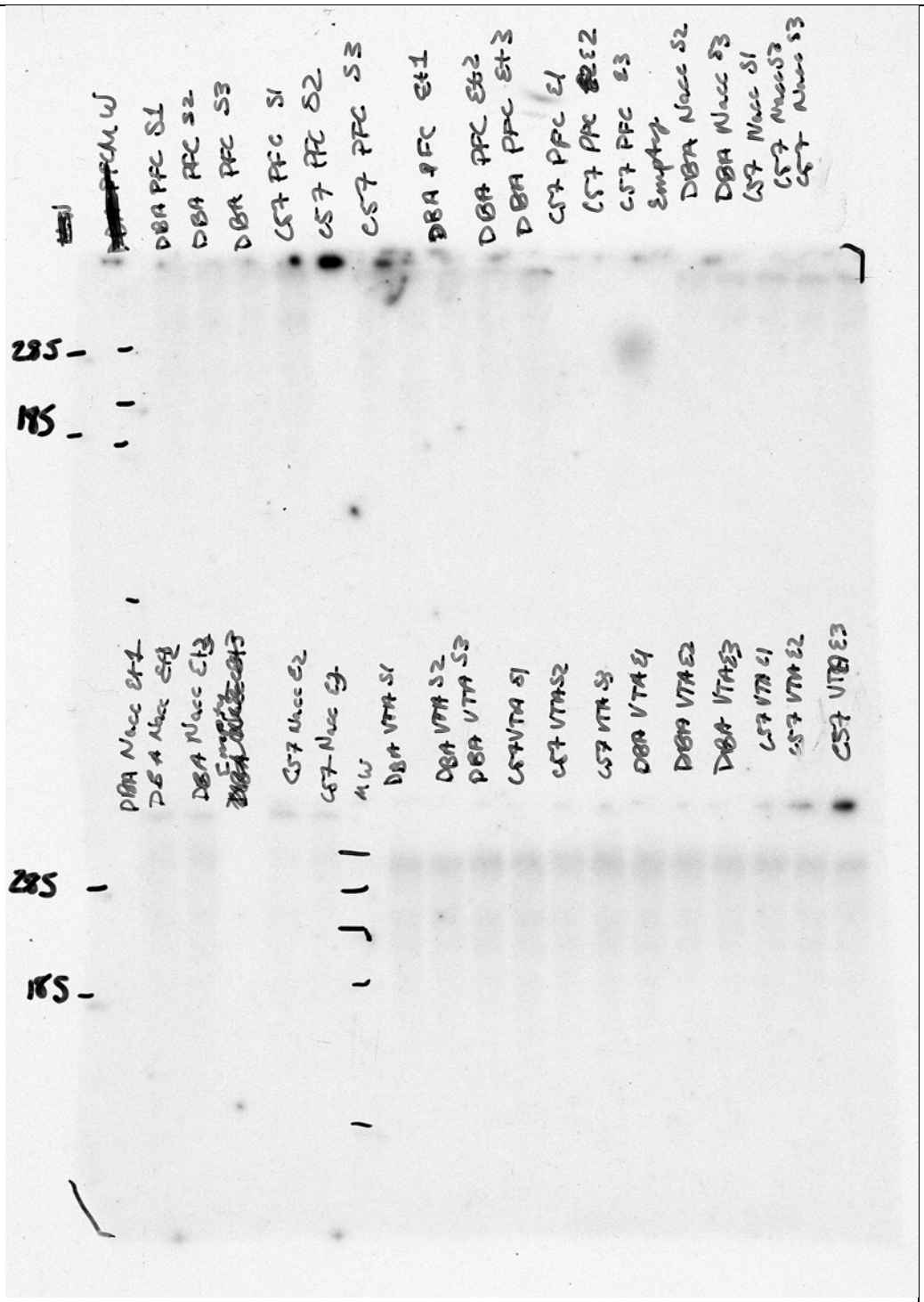
PCR was done in the three prime untranslated region of PAM in the final 195 nucleotides of the target sequence (primers above); the full Affy target sequence (using the same 3' primer and 5' primer: GTTTCCTACCAGTTCACT); and the first 303 nucelotides of the target sequence, with the previous 5' primer and the 3' primer complementary to the 5' primer of the PCR for the distal end of the Affymetrix sequence. PCR was run on genomic DNA from C57 and DBA mice from the prefrontal cortex and cleaned cDNA from saline treated C57 and DBA mice from the prefrontal cortex. cDNA was created from total RNA cleaned twice, using DNAse I. cDNA synthesis was then done with the iScript reaction kit.

Results

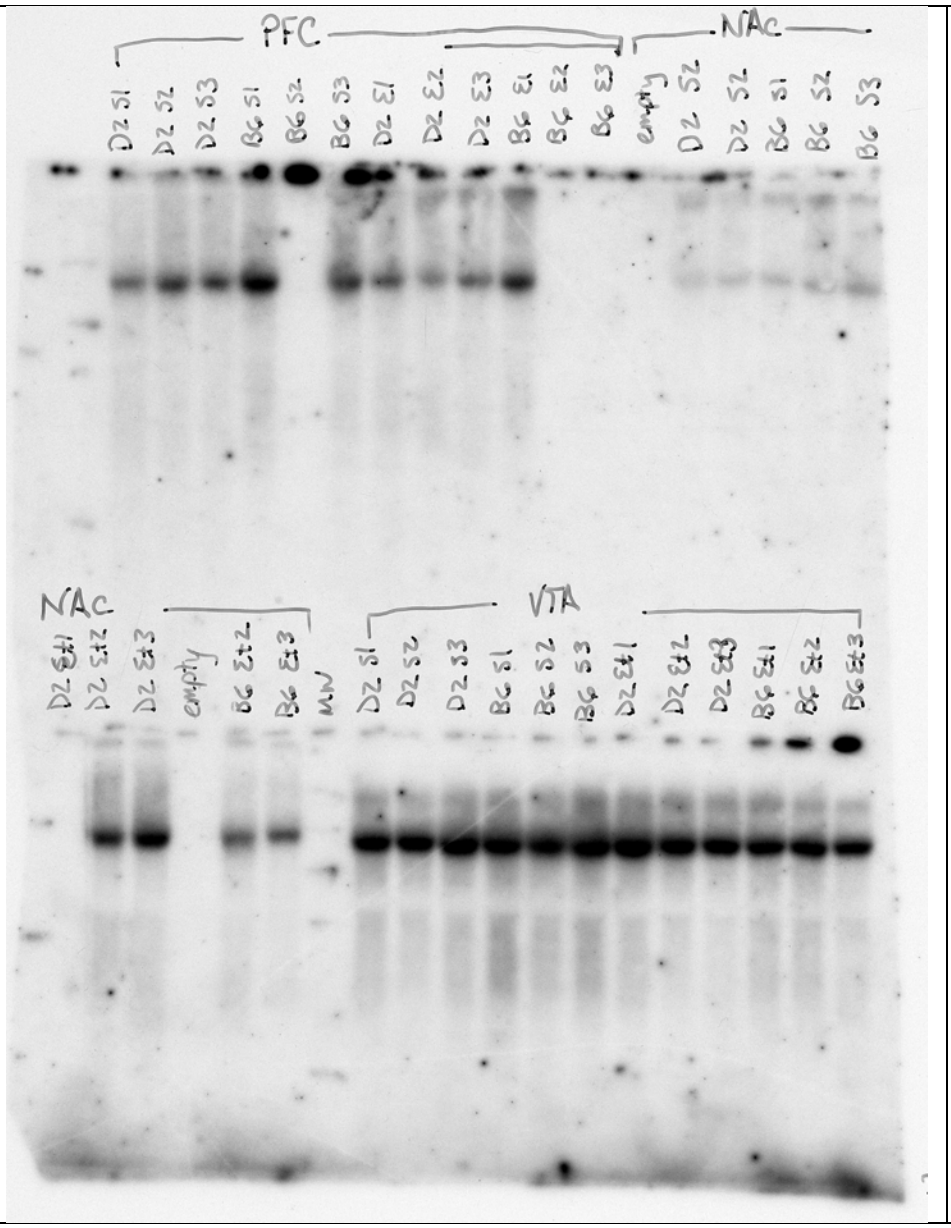
Splicing and Genomic Variants of PAM

Comparison of the DBA and C57 genome sequences revealed no SNPs or variations in the Affymetrix probe areas, ruling out SNPs as a cause for the perceived difference.

Northern blotting with probes from the Affymetrix region and the coding region revealed that there was at least one other splice variant in PAM, that did not contain the Affymetric region.



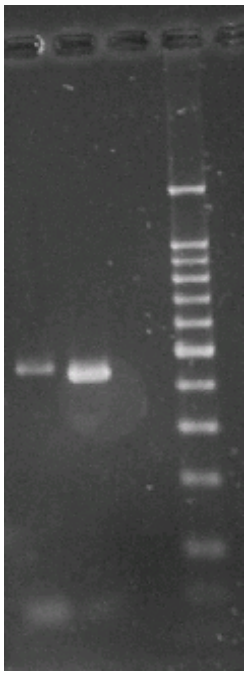
Northern Blot, probed with Affymetrix sequence



Northern blot probed with sequence from PAM Exons two and three

BLAST analysis between mouse and rat PAM also suggests a high homology between them, ranging between 73 and 100% for the 22 exons of PAM (data not shown).

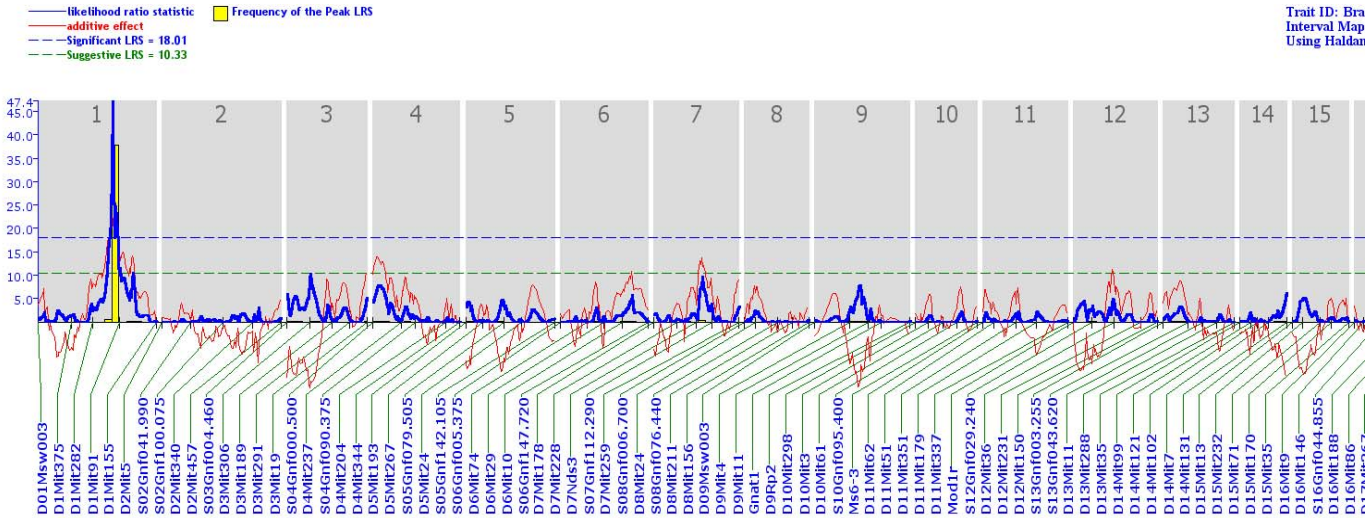
Although MotifFinder did not find multiple consensus sequences for polyadneylation in the 3' UTR, suggesting that there is not a splice variant that ends in the middle of the 3' UTR, size differences in PAM do appear important: PCR in the Affymetrix region appears to confirm the different basal levels of expression, however it also reveals a small size difference between the two strains, present in both cDNA and genomic DNA.(data not shown) (below: C57 cDNA; DBA cDNA against a 1000 nt ladder, probed for the full Affy sequence.)



Further investigation localizes this size difference to the distal 303 nucleotides of the Affymetrix sequence. (Picture not available due to technological difficulties, but will be furnished upon request.)

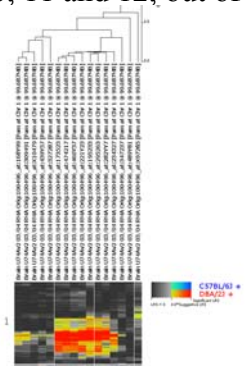
Connecting PAM to Ethanol

WebQTL cluster tree analysis shows a linkage to mouse chromosome one, where PAM sits, and also where Aldehyde Dehydrogenase 1 and other genes that have been connected with alcohol drinking behaviors are situated (Miles, data unpublished).



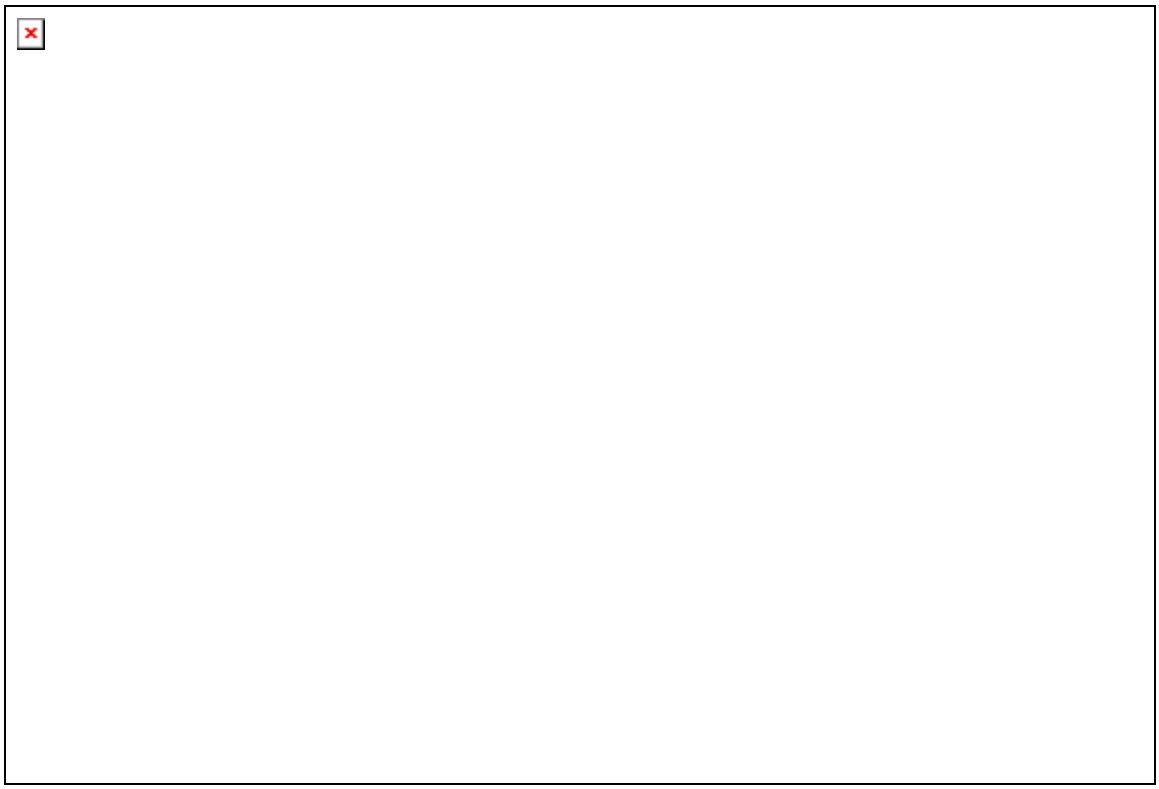
(the large blue peak represents a QTL).

However, further investigation reveals that this linkage is only occurring with probes 1, 4, 5, 6, 7, 8, 9, 10, 11 and 12, out of 13, as shown by the cluster diagram below.



(the dark orange area represents the QTL – probes on the far left and right do not show linkage).

The probes that do not show the linkage, have a higher binding affinity, but do not show a difference between C57 and DBA mice.



(the black line represents C57 mice, and the cyan line DBA mice. The PDNN value represents the affinity with which DNA bound to the probe.) However, there was no significant difference between non-intentional mouse mRNA products that might bind to one set of probes over another.

Although there were no statistical significant gene ontology categories provided by EASE, literature searching revealed a number of possible pathways in which PAM could affect ethanol-drinking behavior (as discussed below.)

Discussion

We set out to discover whether the Affymetrix microarray result suggesting that there were higher levels of expression in PAM in non-ethanol preferring mice. Further, if PAM was more highly expressed in mice that drank less alcohol, we were looking to discover a method by which PAM's expression connected to ethanol drinking.

PCR using cDNA appears to confirm the difference in PAM expression levels between C57 and DBA. In addition, nature language processors, such as Chilibot and Bibliosphere suggest a mechanism for PAM to alter ethanol-drinking behavior. PAM is responsible for amidation of Neuropeptide Y (NPY) and this amidation is necessary for NPY biological activity (9). NPY has been linked to decreased ethanol intake – providing a mechanism for high levels of PAM to cause the DBA phenotype (10). Other possible mechanisms for PAM's influence with ethanol preference exist but are too putative to be within the scope of this paper.

However, equally interesting is the size difference between the genomic PAM in C57 and DBA. In addition to the size difference being present in genomic DNA. Because of the varied performance of probes in the distal end of PAM, it is possible that a

short insertion or deletion is affecting the stability of PAM mRNA. Most likely, an insertion into the C57 genomic sequence between probes 12 and 13, increases the mRNA stability. This would explain why the probes at the distal end of PAM do not show significantly more DBA than C57, and addresses that the difference in expression is made up from increased C57 expression, rather than decreased levels of DBA mRNA. This would also explain why the distal probes do not show a QTL, as intact mRNA of the distal end would not correlate very well to parent strain.

Further, new data suggests that Affymetrix MU74Av2 chips show a very bimodal expression level across all inbred strains of mice (unpublished data). For any given strain of mouse, the average difference value either approximates 300 or 2000 (average difference values are never above 10^4, making this difference quite significant.) A possible interpretation of our data is that the insert in C57, in addition to preserving stability, is also responsible, in some manner, for the decreased expression level of PAM. Combined with the data across all strains, it appears to suggest that PAM expression is controlled by this genomic variation; an idea which demands further exploration before it can be considered a serious conclusion. If this were to be the case, however, it also suggests that providing the shorter version to PAM to certain neuronal areas, thereby increasing PAM activity would be a possible treatment for alcohol abuse.

Acknowledgements

I would like to acknowledge Jennifer Wolstenholme for the creation of riboprobes and hybridizations of Northern Blots, Dr. Robnet T. Kerns for statistical analysis of microarray data, and Dr. Michael Miles for supervision and guidance in this project. This project was funded through the Bioiformatics and Bioengineering Summer Institute.

References

1. Castellano C, Filibreck L, Oliferio A. 1976. Effects of herion, alone or in combination with other drugs, on the locomotor activity in two inbred strains of mice. Psychopharmacology 26;49(1):29-31.
2. Ouafik L, Stoffers D, Campbell T, Johnson R, Bloomquist B, Mains R, Eipper B. 1992. The multifunctional peptidylglycine α-amidating monooxygenase gene: exon/intron organization of catalytic, processing and routing domains. Molecular Endocrinology:1571-1584
3. Eipper B, Green C, Campbell T, Stoffers D, Keutmann H, Mains R, Ouafik L. 1992. Alternative splicing and endoproteolytic processing generate tissue-specific forms of pituitary peptidylglycine α-amidating monooxygenase (PAM). J Biol Chem 267:4008-4015
4. Stoffers D, Green C, Eipper B. 1989. Alternative mRNA splicing generates multiple forms of peptidyl-glycine α-amidating monooxygenase in rat atrium. Proc Natl Acad Sci USA 86:735-739
5. Wang J, Williams R, Manly K. 2003. WebQTL: web-based complex trait analysis. Neuroinformatics 1:299-308.
6. Kerns R, Zhang L, Miles M. 2003. Application of the S-score algorithm for analysis of oligonucleotide microarrays. Methods 31(4):274-81.

7. Zhang L, Miles M, Aldape K. 2003. A model of molecular intereactions on short oligonucleotide microarrays. Nat Biotechnol 21(7):818-21.
8. Hosack D, Dennis G, Sherman B, Lane HC, Lempicki R. 2003. Identifying biological themes within lists of genes with EASE. Genome Biology 4(10):R70.
9. Milgram S, Chang E, Mains R. 1996. Processing and routing of a membrane-anchored form of proneuropeptide Y. Mol Endrocinol 10(7):837-46.
10. Gilpin N, Stewart R, Murphy J, Li T, Badia-Elder N. 2003. Neuropeptide Y reduces oral ethanol intake in alcohol-preferring (P) rats following a period of imposed ethanol abstinence. Alcohol Clin Exp Res 27(5):787-94.