Published in final edited form as:

Addict Biol. 2011 July ; 16(3): 393–404. doi:10.1111/j.1369-1600.2010.00254.x.

Using the PhenoGen Website for “*In Silico*” Analysis of Morphine-Induced Analgesia: Identifying Candidate Genes

Paula L. Hoffman¹, Beth Bennett¹, Laura M. Saba¹, Sanjiv V. Bhav¹, Phyllis J. Carosone-Link¹, Cheryl K. Hornbaker¹, Katerina J. Kechris¹, Robert W. Williams², and Boris Tabakoff¹

¹ University of Colorado Denver, School of Medicine, Department of Pharmacology, Aurora CO USA 80045

² University of Tennessee Health Science Center, Department of Anatomy and Neurobiology, Memphis, TN 38163

Abstract

The identification of genes that contribute to polygenic (complex) behavioral phenotypes is a key goal of current genetic research. One approach to this goal is to combine gene expression information with genetic information, i.e., to map chromosomal regions that regulate gene expression levels. This approach has been termed “genetical genomics”, and, when used in conjunction with the identification of genomic regions (QTLs) that regulate the complex physiological trait under investigation, provides a strong basis for candidate gene discovery. In this paper, we describe the implementation of the genetical genomic/phenotypic approach to identify candidate genes for sensitivity to the analgesic effect of morphine in BXD recombinant inbred mice. Our analysis was performed “*in silico*”, using an online interactive resource called PhenoGen (<http://phenogen.ucdenver.edu>). We describe in detail the use of this resource, which identified a set of candidate genes, some of whose products regulate the cellular localization and activity of the mu opiate receptor. The results demonstrate how PhenoGen can be used to identify a novel set of genes that can be further investigated for their potential role in pain, morphine analgesia and/or morphine tolerance.

Keywords

analgesia; candidate genes; gene expression; morphine; Phenogen; QTL

Introduction

Complex behavioral and physiological phenotypes are influenced by genetic and environmental factors. The identification of the genes that underlie such polygenic traits (“candidate genes”) is a key goal of genetic research. These genes can contribute to the phenotype through variations in the sequence of their coding regions, leading to changes in function of the protein products of the genes. However, in many cases, variations in the expression levels of the genes, i.e., levels of gene transcripts, have been identified as underlying behavioral or disease phenotypes (Schadt et al., 2005). The advent of DNA

Corresponding author: Paula L. Hoffman, Department of Pharmacology, University of Colorado Denver School of Medicine, PO Box 6511, Mail Stop 8303, Aurora, CO 80045-0511, USA, telephone: 303-724-3628, fax: 303-724-3666, paula.hoffman@ucdenver.edu.

Author Contributions: PLH, BB, SVB, LMS, CKH, KJK and BT drafted the manuscript; PLH, BB, LMS, PJC-L, KJK and SVB analyzed the data; CKH and LMS designed and maintained the website; RWW provided information regarding GeneNetwork; BT was responsible for study concept.

microarray technology allows the investigator to simultaneously measure the levels of hundreds or thousands of mRNA transcripts in relevant tissues, but it is often difficult to identify the specific transcripts that are the products of “candidate genes”. One way to address this issue is to combine microarray data with genetic mapping information. Relative transcript levels are treated as quantitative traits in segregating populations, allowing the chromosomal regions that regulate transcript levels to be mapped as expression quantitative trait loci (eQTLs). One can also examine the behavioral or physiologic phenotype across strains of animals or F₂ offspring with known genetic characteristics, to map behavioral (b) QTLs. The identification of eQTLs has been called “genetical genomics” (Jansen and Nap, 2001), and has been successfully used to construct regulatory gene networks in various organisms (Chesler et al., 2005; Lu et al., 2008; Schadt et al., 2005). The identification of common genomic regions (overlapping eQTLs and bQTLs) that regulate not only the complex behavioral/physiological phenotype being studied, but also the expression of genes whose transcript levels are correlated with the phenotype, can be referred to as a “genetical genomic/phenotypic” approach, and provides a strong basis for candidate gene discovery (Chesler et al., 2005; Hu et al., 2008; Tabakoff et al., 2008).

We have developed an online, interactive resource called PhenoGen (<http://phenogen.ucdenver.edu>) that enables researchers to mine data from microarray expression experiments and implement the genetical genomic/phenotypic approach described above (Bhave et al., 2007). The user of the website can upload his or her own microarray data, and/or use microarray data available on the website, to determine differential expression of genes and correlate gene expression with a behavioral phenotype. By locating the sites in the genome where the eQTLs and/or the genes coding for the correlated mRNA levels reside, the user can identify candidate genes based primarily on the overlap of the location of the candidate gene eQTLs and the QTL (bQTL) locations for the behavioral/physiologic trait of interest.

In this paper, we demonstrate the use of the PhenoGen website to identify candidate genes that influence the phenotype of sensitivity to morphine analgesia against thermal pain in mice. Although opiates are a mainstay of pain management, sensitivity both to pain and to opioid analgesia varies substantially among individuals, and at least part of this variation can be attributed to genetic influences (Campa et al., 2008; Fillingim et al., 2005; Kasai et al., 2008; Lacroix-Fralish and Mogil, 2009; Mogil, 1999). Studies of knockout and mutant mice have identified several genes in the opiate receptor signaling pathway that can affect the response to morphine (Lacroix-Fralish and Mogil, 2009), and there is also evidence that DNA polymorphisms that affect the expression of the morphine receptor gene in mice are associated with differences in sensitivity to morphine analgesia (Ikeda et al., 2005; Shigeta et al., 2008).

The genetical genomic/phenotypic approach can be used, in addition to the more targeted approaches described above, to identify candidate genes for morphine analgesia in an unbiased manner. QTLs have been mapped for sensitivity to morphine-induced analgesia in BXD recombinant inbred (RI) mice (derived from the cross between inbred C57BL/6 and DBA/2 mice) (Bergeson et al., 2001). Since the gene for the mu opioid receptor is located within one of these QTLs, it was logically suggested to be a primary candidate gene for sensitivity to morphine analgesia (Belknap et al., 1995; Bergeson et al., 2001; Uhl et al., 1999). However, our analysis, which utilizes the previously-published phenotypic data for sensitivity to morphine-induced analgesia, but now analyzed in combination with brain gene expression levels and eQTL data available on the PhenoGen website, illustrates how an “*in silico*” approach can identify novel candidate genes for morphine analgesia and provide new targets for further investigation.

Materials and Methods

The analyses described in this report were performed “*in silico*”, using microarray data that are available on the PhenoGen website (<http://phenogen.ucdenver.edu>). The Supplementary Methods provide a detailed description of the use of the website, including a step-by-step guide for this particular analysis. The gene expression data were obtained from whole brains of BXD RI mice, using methods described on the PhenoGen website and in previous publications (Hu et al., 2008; Tabakoff et al., 2008). We initiated our analysis by using the precompiled “Public BXD RI Mice” dataset, which contains normalized and quality-controlled expression data from individual whole brain samples from 4–6 biological replicates (mice) per strain (see Supplementary Methods for information on renormalization of the dataset to include selected strains). The inclusion of multiple arrays per strain, with a sample from one individual per array, allows accurate calculation of genetic variance and biological variance (heritability) for each transcript, and increases power for statistical analyses. The measure of heritability gives insight into the strength of genetic control of transcript expression, i.e., correlation of expression level with phenotype represents a true genetic relationship, rather than an association with environmental effects. This relationship has important implications for the relevance of a transcript/protein for drug targeting or disease characterization. Furthermore, with replicate samples within strains, one can evaluate not only mean transcript expression values, but also within-strain variance across strains. Finally, for quality control purposes, outlier arrays can be easily and confidently identified by comparison to other arrays within a strain.

The behavioral data and behavioral (phenotypic) QTLs were published by Belknap and colleagues (Bergeson et al., 2001), and additional detailed phenotypic data for use in this analysis were provided by Dr. John Belknap (personal communication). In brief, morphine-induced antinociception was measured using a hotplate assay, in which animals were injected with 16 mg/kg morphine sulfate, and, 30 min later, were placed on a heated metal plate. Latency to hind paw lift was measured and corrected for latency following a saline injection. Brain gene expression data from 23 of the 25 BXD RI strains used in the behavioral analysis are available on the PhenoGen website, and were used for correlation of gene expression levels with sensitivity to morphine analgesia in the BXD RI strains (see Supplementary Methods).

Creation of Candidate Gene List

The list of candidate genes contributing to variability in sensitivity to the antinociceptive effect of morphine was created using multiple filters that can be implemented on the PhenoGen website. This procedure is described in the Supplementary Methods, and Figure 1 shows a flow chart for using the PhenoGen website. It is important to note that the Affymetrix arrays used to collect the gene expression data often contain more than one probeset targeted to a particular gene, and the analysis and filtering procedures described here are based on probesets, rather than on genes. It is possible that more than one probeset that is targeted to a particular gene can pass the filters and be represented in the final “gene” list, but it is also possible that not all probesets that are targeted to a given gene may pass all of the filters. The PhenoGen output allows the investigator to determine whether there are probesets targeted to a gene in the final list that have not passed the filters (because of alternative splicing issues or for other reasons) (this characteristic of the website is described in detail in the Supplementary Methods). The analysis on the PhenoGen website includes a probe mask that eliminates Affymetrix probes that contain SNPs found in C57BL/6, DBA/2 or other inbred strains of mice, and probes that are targeted incorrectly to the mouse genome.

The methodological steps used in our analysis on the PhenoGen website are briefly described here (Figure 1) and are described in more detail and illustrated in the Supplementary Methods. After re-normalization of the public BXD RI dataset to include only the BXD RI strains for which phenotype data were available, Affymetrix “control” probesets were eliminated from the dataset. Next, probesets that were expressed in brain (called “present” according to the Affymetrix MAS 5.0 algorithm (Affymetrix, 2004)) in 50% of samples in each strain were retained. The next filter that was implemented for these remaining probesets was a heritability filter, to ensure that the genes represented in the final candidate gene list show a strong genetic influence on expression levels. Probesets were eliminated if their heritability of expression in the BXD RI panel was lower than 0.50. The remaining probesets were used in a Pearson product-moment correlation analysis of gene expression levels with sensitivity to morphine antinociception in the BXD RI strains. This filter retained probesets with an unadjusted correlation p value of < 0.05 . The next filter was to identify eQTLs and limit probesets to those that had an eQTL that overlapped the location of a behavioral (phenotypic) QTL for morphine antinociception, obtained from Bergeson et al. (2001). As described in our previous work, the rationale for using this filter is that if a gene contributes to a complex behavior through variation in its expression levels, areas of the genome that regulate those expression levels (eQTLs) should be represented within areas of the genome that contribute to the regulation of the behavior (behavioral or phenotypic QTLs [bQTLs or pQTLs]). Transcripts with eQTL/bQTL overlap are considered to represent likely candidate genes for the particular behavior (Hu et al., 2008; Tabakoff et al., 2008). The determination of eQTLs was described in our earlier work (Hu et al., 2008; Saba et al., 2006; Tabakoff et al., 2008), and the eQTL locations (obtained using whole brain gene expression data from BXD RI mouse strains), including 95% confidence intervals for eQTLs with empirical p values < 0.1 , are available on PhenoGen. The location of an eQTL was defined as the location of the maximum LOD score for each probeset, and eQTLs with p values < 0.1 were used for this analysis. The definition of eQTL/bQTL overlap was that a probeset had a significant or suggestive ($p < 0.1$) eQTL with a 95% confidence interval that overlapped one of the bQTL regions (Tabakoff et al., 2008).

Results

There were 15,766 probesets that were considered to be expressed in brains of all of the BXD RI strains, based on the “present” call by MAS 5.0 (Affymetrix, 2004). Of these probesets, 12,755 (81%) passed the heritability filter. There were 303 probesets whose expression levels correlated with the behavioral phenotype at the $p < 0.05$ level. After applying the eQTL/bQTL overlap filter, the resulting gene list included 14 unique genes. One of these genes did not have any annotation on NCBI, and is not included in the further discussion. Of the 13 remaining transcripts shown in Table 1, 8 were positively correlated with sensitivity to morphine antinociception, and 5 were negatively correlated. The chromosomal locations of the candidate genes, as well as the location of the bQTLs, and of the eQTLs determined using the PhenoGen website, are also shown in Table 1. Four of the candidate genes are posited to be regulated proximally (chromosomal location of the gene is within the eQTL’s 95% confidence limits), while the rest are regulated distally (the eQTL is on a chromosome different from that where the gene is physically located). The Discussion section includes an outline of the function of some of these genes and their potential role in sensitivity to morphine analgesia.

The PhenoGen website contains a link to the Allen Brain Atlas. Since the analysis described here was performed using whole brain gene expression data, the Allen Brain Atlas was used to determine brain regional localization of the candidate genes for morphine analgesia, including possible colocalization of the transcripts, which allows one to consider functional interactions among the genes/gene products. This atlas uses *in situ* hybridization with

C57BL/6 mouse brains to localize and estimate the relative quantity of mRNA levels in various brain regions, and care was taken in the construction of the Atlas to determine that signal intensity and quality were consistent across multiple probe syntheses and different brain samples (http://mouse.brain-map.org/pdf/ISH_Platform_Controls.pdf). Based on the results in the atlas, many of the transcripts showed relatively high levels of expression throughout brain, and most transcripts showed colocalized expression in at least some large brain regions, such as cerebral cortex, hippocampus, cerebellum and striatum. However, in this instance, because there is substantial information available on neuronal pathways mediating pain and analgesia, and because the phenotype being studied involves the action of morphine, we included expression of the mu opiate receptor gene (*Oprm1*) in our analysis of transcript localization, and focused this analysis on the candidate genes with gene products that may interact with the mu receptor. (It is important to note, however, that *Oprm1* expression itself was not significantly correlated with sensitivity to morphine antinociception in the BXD RI mice). We also focused on brain circuits involved in pain transmission. As illustrated in Figure 2 and Table 2, we noted the co-localized expression of several of the candidate genes in these pain transmission regions.

Discussion

In this manuscript and the accompanying Supplementary Methods, we have described in detail the use of the website PhenoGen (<http://phenogen.ucdenver.edu>) for identification of candidate genes that contribute to complex physiological/behavioral traits through variation in their brain expression (mRNA) levels. The process outlined is a systematic, global analysis of the correlation of brain gene expression levels with the phenotype across a panel of genetically characterized recombinant inbred strains of mice. The correlated transcripts are subjected to several filters, including the critical step of determining common QTLs for control of transcript expression and phenotype (eQTL overlap with bQTL). This particular process is an essential component of candidate gene identification, and is a key aspect of the design of PhenoGen.

A key feature of PhenoGen is that users can upload their own microarray data, or use data already resident on the site, for this type of analysis. Users have many tools for analyzing raw data, including quality control, a choice of normalization procedures, several filtering methods for eliminating data from non-informative or noisy probesets, the ability to choose appropriate statistical methods for data analysis, as well as access to comprehensive annotation and literature search capabilities. The use of these PhenoGen tools is described by example in the Supplementary Methods, and in the User's Manual that can be downloaded from the website.

There are a number of databases that investigators can use to assist in various aspects of gene expression data storage and mining (e.g., (Chesler et al., 2005; Galperin and Cochrane, 2009; Gentleman et al., 2004; Mailman et al., 2007; Saal et al., 2002; Swertz et al., 2010)). One relatively well-known database is GeneNetwork (www.genenetwork.org) (Chesler et al., 2005). GeneNetwork is designed primarily as a web service for exploratory and statistical analysis of large published phenotype and genome datasets, and includes data from several species (see Supplementary Discussion). GeneNetwork includes extensive phenotype data extracted from the literature and submitted by users, which makes it practical to compare data on drug responses with gene expression patterns. Gene expression data are entered into GeneNetwork after they have been shepherded through a system like PhenoGen that has extensive capabilities for normalization and quality control. A comparison of the brain gene expression datasets and some of the tools for data analysis available on PhenoGen and GeneNetwork is shown in Table 3, and more detailed information on features provided by each site is outlined in the Supplementary Discussion

(description of GeneNetwork provided by Dr. Robert W. Williams). Both of these websites focus to a large extent on correlations of behavioral phenotype with gene expression levels in recombinant inbred and inbred panels of mice and rats, and on QTL analyses, as a means to identify candidate genes for complex traits. What distinguishes PhenoGen, in addition to the tools for raw expression data analysis described above, is that the user can not only perform their own quality control analysis of the gene expression data available but can also remove arrays that do not meet the user's threshold for quality. The user also has the opportunity to select the most appropriate test(s) needed to perform a statistically rigorous analysis of gene expression, and of phenotype-gene expression correlations (with on-line help and a User's Manual which describe and justify each test). Importantly, the datasets resident on PhenoGen include biological replicates, and are controlled for variables such as the age and sex of the animals, making them amenable to in-depth statistical analyses. The use of GeneNetwork can be complementary to the PhenoGen analyses. For example, a user could focus on particular candidate genes identified on PhenoGen, and assess their expression levels and correlations with phenotype across brain regions, or even other tissue types (e.g., liver or lung) on GeneNetwork. However, differences in the datasets on the two websites (outlined in Table 3) need to be taken into consideration if such comparisons are attempted.

PhenoGen is organized primarily to allow for genetical genomic/phenotypic analysis that includes the determination of bQTL/eQTL overlap as a key aspect of candidate gene identification. However, the site also provides other tools for gene expression analysis, such as promoter analyses (oPOSSUM and MEME), and co-expression and clustering analyses, including graphical tools for visualization of these results. We describe the use of these tools in Supplementary Methods, but the focus of the current work is to demonstrate how PhenoGen can be used to identify candidate genes for sensitivity to morphine analgesia, using the genetical genomic/phenotypic approach.

Recent review articles have reported on several genes and gene products that influence sensitivity to morphine-induced analgesia in animals (Kasai et al., 2008; Lacroix-Fralish and Mogil, 2009). Many of the studies use knockout mice or animals in which the expression of a specific gene has been "knocked down" (Kasai et al., 2008), including mice which are null mutants for the various forms of the opioid receptors (μ , δ and κ). Given the importance of the opiate receptor for initiating the signaling that mediates the analgesic response to morphine, it may not be surprising that knocking down or knocking out the genes that code for the opiate receptors themselves, as well as certain molecules known to be downstream effectors of the morphine receptor involved in the intracellular signaling processes, would affect the analgesic response to morphine. However, these types of manipulations do not necessarily reflect the naturally-occurring genetic variations that contribute to differences in response to morphine analgesia.

In our analyses of the BXD RI mice, we did not observe a significant correlation of μ opiate receptor expression (*Oprm1* mRNA levels), or transcript levels for the δ or κ opiate receptors, with sensitivity to morphine-induced analgesia. It has previously been noted that strain order for sensitivity to morphine analgesia is related to the nature of the pain being inhibited by the drug, rather than to the drug itself (Lacroix-Fralish and Mogil, 2009). These findings support the conclusion that it cannot be only the level of expression or activity of the morphine receptor that contributes to variation in sensitivity to morphine analgesia, and reinforce the idea that genes that influence sensitivity to particular types of pain may also play a role in mediating differential responses to morphine analgesia.

μ opioid receptors are regulated by receptor desensitization and endocytosis, with subsequent recycling to the plasma membrane (resensitization) (Bailey and Connor, 2005;

Martini and Whistler, 2007). A number of the candidate genes/gene products that we identified can potentially affect the endocytic recycling of opiate receptors. These include the protein products of *Spag9*, *Pacsin1* and *Phactr2*. The protein product of *Spag9*, JLP, is a scaffolding protein that activates the p38 MAP kinase signaling pathway (Kelkar et al., 2005; Lee et al., 2002), which has previously been implicated in morphine receptor endocytosis (Macé et al., 2005) and development of tolerance to morphine-induced analgesia (Cui et al., 2006; Wang et al., 2009). The protein product of *Pacsin1* is a Src homology 3-domain-containing protein that also plays a role in endocytosis, as well as in regulation of actin dynamics (Wasiak et al., 2001). The protein product of *Phactr2* is a member of a family of proteins that interact with protein phosphatase 1 (PP-1) and actin, and may direct PP-1 toward neuronal substrates associated with the actin skeleton (Allen et al., 2004). *Phactr2* is of particular interest, given the suggested role of phosphatase activity in the mechanism of both the antinociceptive effect of morphine and morphine tolerance (Gabra et al., 2007; Moncada et al., 2003). In fact, another PP-1 targeting protein, spinophilin, has been implicated in sensitivity to the analgesic effect of morphine and in morphine tolerance and dependence (Charlton et al., 2008).

The protein product of another candidate gene, *Fuca2*, is a fucosidase, an enzyme that can regulate the addition and removal of fucose to brain glycoproteins. The mu receptor is a heavily glycosylated protein, with complex carbohydrate moieties, N-linked to asparagine (Liu-Chen et al., 1993). The single nucleotide polymorphism, A118G, in the coding region of the mu receptor, removes a potential extracellular glycosylation site, and affects the function of the mu receptor (Kroslak et al., 2007). This polymorphism has been reported to affect pain sensitivity in humans (Fillingim et al., 2005), and has also been reported to be associated with altered opiate efficacy in several studies with human subjects (Kasai et al., 2008). The equivalent polymorphism (SNP) is not found in C57BL/6 vs DBA/2 mice, but in a recent report, knock-in mice that contained the SNP A112G, which is equivalent to the human A118G SNP, displayed reduced morphine antinociception, compared to wild-type mice (Mague et al., 2009). One can speculate that fucosidase could modulate mu receptor A118 (or other asparagine residue) glycosylation, such that differences in the expression of this enzyme could indirectly alter mu receptor function and/or sensitivity to morphine analgesia.

Although the interactions among the products of the candidate genes can be discussed based on known protein functions, these candidate genes were identified using whole mouse brain gene expression analysis. If the transcripts and/or gene products are not expressed in the same brain regions (ideally, in the same cells), then these theoretical protein interactions are not likely to occur. Similarly, interactions between candidate gene products and the mu receptor depend on co-localization of these genes/gene products. We assessed the brain regional localization and co-localization of candidate transcript expression, as well as morphine (mu) receptor mRNA expression, using the Allen Brain Atlas (<http://www.brain-map.org>) accessed through the PhenoGen website. We focused our search for regional colocalization of gene expression on known CNS pain pathways (Campbell and Meyer, 2006; Finnerup et al., 2007; Hunt and Mantyh, 2001). We found that many of the identified candidate genes discussed above, whose products may affect mu receptor function and/or internalization, as well as the mu opiate receptor gene itself, displayed expression that co-localized in the thalamus, parabrachial nucleus and hypothalamus (Figure 1, Table 2). The thalamus and parabrachial nucleus are regions that receive input from sensory neurons, while the hypothalamus is a target area for neurons arising from the parabrachial nucleus, that carry information on the emotional concomitants of pain. Thus, our discussion of the potential interactions of the candidate gene products with the mu receptor, which in turn modulate the animal's analgesic response to morphine, can be limited to particular brain regions involved in pain mediation. This component of our data analysis demonstrates the

usefulness of combining analysis of whole brain gene expression data with anatomical localization information for candidate genes that can be obtained from sites such as the Allen Brain Atlas.

Some of the protein products of the other candidate genes may also influence the response to morphine either directly, or by influencing the development of tolerance to morphine. The product of *Plagl1* is a zinc finger protein that can induce the expression of the type 1 (PAC1) receptor for pituitary adenylyl cyclase activating protein (PACAP) (Hoffmann et al., 1998; Rodriguez-Henche et al., 2002). PACAP has been shown to reduce the analgesic effect of morphine and to increase chronic morphine tolerance (Mácsai et al., 2002).

An important issue in the type of analysis described here is the behavioral phenotype chosen for study. It has been found in many studies that there is a strong negative genetic correlation between pain sensitivity and analgesic sensitivity (Lacroix-Fralish and Mogil, 2009), and there is evidence that the protein products of *Pparg1a* (PGC-1 α) and *Mgst3* can be related to pain sensitivity (Fehrenbacher et al., 2009; Finck and Kelly, 2006; Napimoga et al., 2008). Transcriptional control elements have also been proposed to play a role in sensitivity or tolerance to opiates and other drugs (Bailey and Connor, 2005). We identified several candidate genes that regulate transcription and polyadenylation of mRNA precursors (*Zcchc24*, *Cstf3* (Bai et al., 2007)), as well as *Spnr* and *Odz2*, which are involved in neuronal remodeling and neurite outgrowth (Severt et al., 1999; Young and Leamey, 2009; Zhou et al., 2003).

The finding that known functions of many of the identified candidate genes fit into cogent functional pathways that can influence sensitivity to pain and/or morphine analgesia, provides reassurance that these genes are “true positives.” However, confirmation of differences in mRNA levels of candidate genes across strains, using other methods such as qRT-PCR, and/or assessment of protein levels by immunoblotting or other measures, is still necessary. The function of the candidate genes in sensitivity to pain and/or morphine analgesia can also be assessed using appropriate null mutant models or methods such as RNA interference for altering the levels of candidate gene expression in selected brain regions.

Overall, the study described here illustrates the use of PhenoGen for the practical application of the genetical genomic/phenotypic approach to an *in silico* search for a subset of candidate genes for a complex physiological phenotype. Such novel sets of candidate genes can then be further investigated for their potential role in pain, as well as in the analgesic response and/or development of tolerance to morphine. An investigator can use the process described herein for most other physiologic/behavioral phenotypes by combining appropriate phenotype data with the gene expression data available on PhenoGen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. John Belknap for providing the detailed behavioral data used in our analysis. This work was supported in part by NIH, NIAAA: R24AA013162 (BT), U01AA016663 (BT), U01AA016649 (PH), U01AA013499 (RWW), U01AA014425 (RWW); NIH, NIDA P20-DA 21131 (RWW); and the Banbury Fund (BT).

References

- Affymetrix. Data analysis fundamentals. Affymetrix, Inc; Santa Clara, CA: 2004. GeneChip expression analysis.
- Allen PB, Greenfield AT, Svenningsson P, Haspelagh DC, Greengard P. Phactrs 1–4: A family of protein phosphatase 1 and actin regulatory proteins. *Proc Natl Acad Sci U S A*. 2004; 101:7187–7192. [PubMed: 15107502]
- Bai Y, Auferin TC, Chou CY, Chang GG, Manley JL, Tong L. Crystal structure of murine CstF-77: dimeric association and implications for polyadenylation of mRNA precursors. *Mol Cell*. 2007; 25:863–875. [PubMed: 17386263]
- Bailey CP, Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol*. 2005; 5:60–68. [PubMed: 15661627]
- Belknap JK, Mogil JS, Helms ML, Richards SP, O'Toole LA, Bergeson SE, Buck KJ. Localization to chromosome 10 of a locus influencing morphine analgesia in crosses derived from C57BL/6 and DBA/2 strains. *Life Sci*. 1995; 57:PL117–124. [PubMed: 7643715]
- Bergeson SE, Helms ML, O'Toole LA, Jarvis MW, Hain HS, Mogil JS, Belknap JK. Quantitative trait loci influencing morphine antinociception in four mapping populations. *Mamm Genome*. 2001; 12:546–553. [PubMed: 11420618]
- Bhave SV, Hornbaker C, Phang TL, Saba L, Lapadat R, Kechris K, Gaydos J, McGoldrick D, Dolbey A, Leach S, Soriano B, Ellington A, Ellington E, Jones K, Mangion J, Belknap JK, Williams RW, Hunter LE, Hoffman PL, Tabakoff B. The PhenoGen informatics website: tools for analyses of complex traits. *BMC Genet*. 2007; 8:59. [PubMed: 17760997]
- Campa D, Gioia A, Tomei A, Poli P, Barale R. Association of ABCB1/MDR1 and OPRM1 gene polymorphisms with morphine pain relief. *Clin Pharmacol Ther*. 2008; 83:559–566. [PubMed: 17898703]
- Campbell JN, Meyer RA. Mechanisms of neuropathic pain. *Neuron*. 2006; 52:77–92. [PubMed: 17015228]
- Charlton JJ, Allen PB, Psifogeorgou K, Chakravarty S, Gomes I, Neve RL, Devi LA, Greengard P, Nestler EJ, Zachariou V. Multiple actions of spinophilin regulate mu opioid receptor function. *Neuron*. 2008; 58:238–247. [PubMed: 18439408]
- Chesler EJ, Lu L, Shou S, Qu Y, Gu J, Wang J, Hsu HC, Mountz JD, Baldwin NE, Langston MA, Threadgill DW, Manly KF, Williams RW. Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. *Nat Genet*. 2005; 37:233–242. [PubMed: 15711545]
- Cui Y, Chen Y, Zhi JL, Guo RX, Feng JQ, Chen PX. Activation of p38 mitogen-activated protein kinase in spinal microglia mediates morphine antinociceptive tolerance. *Brain Res*. 2006; 1069:235–243. [PubMed: 16403466]
- Fehrenbacher JC, Loverme J, Clarke W, Hargreaves KM, Piomelli D, Taylor BK. Rapid pain modulation with nuclear receptor ligands. *Brain Res Rev*. 2009; 60:114–124. [PubMed: 19162071]
- Fillingim RB, Kaplan L, Staud R, Ness TJ, Glover TL, Campbell CM, Mogil JS, Wallace MR. The A118G single nucleotide polymorphism of the mu-opioid receptor gene (OPRM1) is associated with pressure pain sensitivity in humans. *J Pain*. 2005; 6:159–167. [PubMed: 15772909]
- Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest*. 2006; 116:615–622. [PubMed: 16511594]
- Finnerup NB, Sindrup SH, Jensen TS. Chronic neuropathic pain: mechanisms, drug targets and measurement. *Fundam Clin Pharmacol*. 2007; 21:129–136. [PubMed: 17391285]
- Gabra BH, Bailey CP, Kelly E, Sanders AV, Henderson G, Smith FL, Dewey WL. Evidence for an important role of protein phosphatases in the mechanism of morphine tolerance. *Brain Res*. 2007; 1159:86–93. [PubMed: 17582387]
- Galperin MY, Cochrane GR. Nucleic Acids Research annual Database Issue and the NAR online Molecular Biology Database Collection in 2009. *Nucleic Acids Res*. 2009; 37:D1–4. [PubMed: 19033364]
- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini

- AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 2004; 5:R80. [PubMed: 15461798]
- Hoffmann A, Ciani E, Houssami S, Brabet P, Journot L, Spengler D. Induction of type I PACAP receptor expression by the new zinc finger protein Zac1 and p53. *Ann N Y Acad Sci.* 1998; 865:49–58. [PubMed: 9927996]
- Hu W, Saba L, Kechris K, Bhawe SV, Hoffman PL, Tabakoff B. Genomic insights into acute alcohol tolerance. *J Pharmacol Exp Ther.* 2008; 326:792–800. [PubMed: 18550690]
- Hunt SP, Mantyh PW. The molecular dynamics of pain control. *Nat Rev Neurosci.* 2001; 2:83–91. [PubMed: 11252998]
- Ikeda K, Ide S, Han W, Hayashida M, Uhl GR, Sora I. How individual sensitivity to opiates can be predicted by gene analyses. *Trends Pharmacol Sci.* 2005; 26:311–317. [PubMed: 15925706]
- Jansen RC, Nap JP. Genetical genomics: the added value from segregation. *Trends Genet.* 2001; 17:388–391. [PubMed: 11418218]
- Kasai S, Hayashida M, Sora I, Ikeda K. Candidate gene polymorphisms predicting individual sensitivity to opioids. *Naunyn Schmiedeberg's Arch Pharmacol.* 2008; 377:269–281. [PubMed: 17999051]
- Kelkar N, Standen CL, Davis RJ. Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways. *Mol Cell Biol.* 2005; 25:2733–2743. [PubMed: 15767678]
- Kroslak T, Laforge KS, Gianotti RJ, Ho A, Nielsen DA, Kreek MJ. The single nucleotide polymorphism A118G alters functional properties of the human mu opioid receptor. *J Neurochem.* 2007; 103:77–87. [PubMed: 17877633]
- Lacroix-Fralish ML, Mogil JS. Progress in genetic studies of pain and analgesia. *Annu Rev Pharmacol Toxicol.* 2009; 49:97–121. [PubMed: 18834308]
- Lee CM, Onesime D, Reddy CD, Dhanasekaran N, Reddy EP. JLP: A scaffolding protein that tethers JNK/p38MAPK signaling modules and transcription factors. *Proc Natl Acad Sci U S A.* 2002; 99:14189–14194. [PubMed: 12391307]
- Liu-Chen LY, Chen C, Phillips CA. Beta-[3H]fentanyl-labeled mu-opioid receptors: species variations in molecular mass and glycosylation by complex-type, N-linked oligosaccharides. *Mol Pharmacol.* 1993; 44:749–756. [PubMed: 8232225]
- Lu L, Wei L, Peirce JL, Wang X, Zhou J, Homayouni R, Williams RW, Airey DC. Using gene expression databases for classical trait QTL candidate gene discovery in the BXD recombinant inbred genetic reference population: mouse forebrain weight. *BMC Genomics.* 2008; 9:444. [PubMed: 18817551]
- Macé G, Miaczynska M, Zerial M, Nebreda AR. Phosphorylation of EEA1 by p38 MAP kinase regulates mu opioid receptor endocytosis. *EMBO J.* 2005; 24:3235–3246. [PubMed: 16138080]
- Mácsai M, Pataki I, Toth G, Szabo G. The effects of pituitary adenylate cyclase-activating polypeptide on acute and chronic morphine actions in mice. *Regul Pept.* 2002; 109:57–62. [PubMed: 12409215]
- Mague SD, Isiegas C, Huang P, Liu-Chen LY, Lerman C, Blendy JA. Mouse model of OPRM1 (A118G) polymorphism has sex-specific effects on drug-mediated behavior. *Proc Natl Acad Sci U S A.* 2009; 106:10847–10852. [PubMed: 19528658]
- Mailman MD, Feolo M, Jin Y, Kimura M, Tryka K, Bagoutdinov R, Hao L, Kiang A, Paschall J, Phan L, Popova N, Pretel S, Ziyabari L, Lee M, Shao Y, Wang ZY, Sirotkin K, Ward M, Kholodov M, Zbicz K, Beck J, Kimelman M, Shevelev S, Preuss D, Yaschenko E, Graeff A, Ostell J, Sherry ST. The NCBI dbGaP database of genotypes and phenotypes. *Nat Genet.* 2007; 39:1181–1186. [PubMed: 17898773]
- Martini L, Whistler JL. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Curr Opin Neurobiol.* 2007; 17:556–564. [PubMed: 18068348]
- Mogil JS. The genetic mediation of individual differences in sensitivity to pain and its inhibition. *Proc Natl Acad Sci U S A.* 1999; 96:7744–7751. [PubMed: 10393892]

- Moncada A, Cendan CM, Baeyens JM, Del Pozo E. Effects of serine/threonine protein phosphatase inhibitors on morphine-induced antinociception in the tail flick test in mice. *Eur J Pharmacol.* 2003; 465:53–60. [PubMed: 12650833]
- Napimoga MH, Souza GR, Cunha TM, Ferrari LF, Clemente-Napimoga JT, Parada CA, Verri WA Jr, Cunha FQ, Ferreira SH. 15d-prostaglandin J2 inhibits inflammatory hypernociception: involvement of peripheral opioid receptor. *J Pharmacol Exp Ther.* 2008; 324:313–321. [PubMed: 17928570]
- Rodriguez-Henche N, Jamen F, Leroy C, Bockaert J, Brabet P. Transcription of the mouse PAC1 receptor gene: cell-specific expression and regulation by Zac1. *Biochim Biophys Acta.* 2002; 1576:157–162. [PubMed: 12031496]
- Saal LH, Troein C, Vallon-Christersson J, Gruvberger S, Borg A, Peterson C. BioArray Software Environment (BASE): a platform for comprehensive management and analysis of microarray data. *Genome Biol.* 2002; 3:SOFTWARE0003. [PubMed: 12186655]
- Saba L, Bhawe SV, Grahame N, Bice P, Lapadat R, Belknap J, Hoffman PL, Tabakoff B. Candidate genes and their regulatory elements: alcohol preference and tolerance. *Mamm Genome.* 2006; 17:669–688. [PubMed: 16783646]
- Schadt EE, Lamb J, Yang X, Zhu J, Edwards S, Guhathakurta D, Sieberts SK, Monks S, Reitman M, Zhang C, Lum PY, Leonardson A, Thieringer R, Metzger JM, Yang L, Castle J, Zhu H, Kash SF, Drake TA, Sachs A, Lusis AJ. An integrative genomics approach to infer causal associations between gene expression and disease. *Nat Genet.* 2005; 37:710–717. [PubMed: 15965475]
- Severt WL, Biber TU, Wu X, Hecht NB, DeLorenzo RJ, Jakoi ER. The suppression of testis-brain RNA binding protein and kinesin heavy chain disrupts mRNA sorting in dendrites. *J Cell Sci.* 1999; 112 (Pt 21):3691–3702. [PubMed: 10523505]
- Shigeta Y, Kasai S, Han W, Hata H, Nishi A, Takamatsu Y, Hagino Y, Yamamoto H, Koide T, Shiroishi T, Kasai K, Tsunashima K, Kato N, Ikeda K. Association of morphine-induced antinociception with variations in the 5' flanking and 3' untranslated regions of the mu opioid receptor gene in 10 inbred mouse strains. *Pharmacogenet Genomics.* 2008; 18:927–936. [PubMed: 18854775]
- Swertz MA, Velde KJ, Tesson BM, Scheltema RA, Arends D, Vera G, Alberts R, Dijkstra M, Schofield P, Schughart K, Hancock JM, Smedley D, Wolstencroft K, Goble C, de Brock EO, Jones AR, Parkinson HE, Jansen RC. XGAP: a uniform and extensible data model and software platform for genotype and phenotype experiments. *Genome Biol.* 2010; 11:R27. [PubMed: 20214801]
- Tabakoff B, Saba L, Kechris K, Hu W, Bhawe SV, Finn DA, Grahame NJ, Hoffman PL. The genomic determinants of alcohol preference in mice. *Mamm Genome.* 2008; 19:352–365. [PubMed: 18563486]
- Uhl GR, Sora I, Wang Z. The mu opiate receptor as a candidate gene for pain: polymorphisms, variations in expression, nociception, and opiate responses. *Proc Natl Acad Sci U S A.* 1999; 96:7752–7755. [PubMed: 10393893]
- Wang Z, Ma W, Chabot JG, Quirion R. Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia. *FASEB J.* 2009; 23:2576–2586. [PubMed: 19299480]
- Wasiak S, Quinn CC, Ritter B, de Heuvel E, Baranes D, Plomann M, McPherson PS. The Ras/Rac guanine nucleotide exchange factor mammalian Son-of-sevenless interacts with PACSIN 1/ syndapin I, a regulator of endocytosis and the actin cytoskeleton. *J Biol Chem.* 2001; 276:26622–26628. [PubMed: 11352907]
- Young TR, Leamey CA. Teneurins: important regulators of neural circuitry. *Int J Biochem Cell Biol.* 2009; 41:990–993. [PubMed: 18723111]
- Zhou XH, Brandau O, Feng K, Ohashi T, Ninomiya Y, Rauch U, Fassler R. The murine Ten-m/Odz genes show distinct but overlapping expression patterns during development and in adult brain. *Gene Expr Patterns.* 2003; 3:397–405. [PubMed: 12915301]

Figure 1. Flow chart for Phenogen use

The various tabs on the Phenogen website, as described in the text and Supplementary Methods, are represented in this figure by the different colors. The area in blue, “Analyze Microarray Data”, represents the unique aspects of PhenoGen that particularly distinguish it from the GeneNetwork website (see Table 3). The dashed lines represent possible entry and exit points for the indicated analyses.

Figure 2. Brain regional expression of *Oprm1* (A) (red), *Phacr2* (B) (blue) and *Spag9* (C) (yellow)
 Data were obtained from the Allen Brain Atlas (<http://www.brain-map.org>). The images were obtained using software (Brain Explorer) available on the website. The background colors (e.g., green for cerebral cortex) are used by the Allen Brain Atlas to delineate brain regions. The figure shows a 2-dimensional representation of the 3-dimensional reconstructions generated by Brain Explorer using data obtained from sagittal brain sections. Localization of transcript expression in cerebral cortex is not shown in these figures, in order to allow visualization of gene expression in other areas, such as parabrachial nucleus and thalamus.

Table 1

Candidate genes for sensitivity to morphine analgesia in BXD RI mice.

Affymetrix ID	Gene Symbol	Gene Name	Correlation Coefficient	Correlation p-Value	Gene Location Chr: Mb	eQTL Location Chr: Mb	eQTL max LOD	eQTL p-Value
1445143_at	Vash1	Vasohibin 1	0.481	0.015	12:88.0	1:182.12	3.25	0.065
1448300_at	Mgst3	Microsomal glutathione S-transferase 3	0.465	0.019	1:169.3	1:168.23	5.45	0.001
1416046_a_at	Fuca2	Fucosidase, alpha-L-2, plasma	-0.406	0.044	10:13.2	10:3.34	5.05	0.001
1426208_x_at	Plagl1	Pleiomorphic adenoma gene-like 1	-0.479	0.015	10:12.9	10:3.34	3.84	0.041
1428432_at	Zcche24	Zinc finger, CCHC domain containing 24	-0.404	0.045	14:26.5	10:30.70	3.51	0.057
1437676_at	Spag9	Sperm associated antigen 9	0.400	0.048	11:93.9	10:30.70	3.54	0.083
1442622_at	Srbp	Spermatid perinuclear RNA binding protein	0.424	0.034	2:37.5	10:28.47	3.30	0.094
1443909_at	Cstf3	Cleavage stimulation factor, 3' pre-RNA, subunit 3	0.427	0.033	2:104.5	10:30.70	3.52	0.029
1444842_at	Kdm5a	Lysine (K)-specific demethylase 5A	-0.496	0.012	6:120.3	10:9.30	3.89	0.022
1449381_a_at	Pacsin1	Protein kinase C and casein kinase substrate in neurons 1	0.487	0.014	17:27.8	10:30.70	3.74	0.072
1452404_at	Phactr2	Phosphatase and actin regulator 2	-0.534	0.006	10:12.9	10:3.34	7.02	<0.001
1454424_at	Odd2	Odd Oz/ten-m homolog 2	0.406	0.044	11:36.5	10:108.80	3.41	0.062
1456395_at	Pparg1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	0.417	0.038	5:51.8	10:30.70	4.09	0.040

bQTL locations for morphine analgesia (Bergeson et al., 2001):

Chromosome:	Mb
1:	0-40
9:	43-90
9:	108-120
10:	0-30

Table 2

Brain regional expression of genes correlated with morphine induced analgesia

Gene symbol	Parabrachial nucleus	Thalamus	Hypothalamus	Somatosensory cortex	Amygdala
<i>Oprm1</i>	++	+++	++	nd	nd
<i>Phactr2</i>	+	+++	++	nd	++
<i>Pacsin1</i>	+++	+++	++++	++++	++++
<i>Spag9</i>	++	+++	++++	++++	++++
<i>Fuca2</i>	+	+	++	++	++

Gene expression data were obtained from Allen Brain Atlas (<http://www.brain-map.org>) and are expressed in arbitrary units to provide qualitative estimate of relative expression levels.

nd = Not Detected

Table 3

Brain Gene Expression *, Phenotype and QTL Information Available on PhenoGen and GeneNetwork.

PhenoGen	GeneNetwork
Gene Expression Data	
<p><u>Precompiled Public Databases (whole brain)</u> BXD RI mice (32 strains) Mouse Diversity Panel (20 strains) HXB/BXH RI rats (29 strains) LXS RI mice (67 strains) (exon arrays are being posted)</p> <p><u>Public Data (whole brain unless otherwise noted)</u> <u>Selected mouse lines:</u> HAFT/LAFT (2 replicate lines); HAP/LAP (3 replicate lines); ILS/ISS <u>Genetically Altered Mice and Crosses</u> WT/AC7 Transgenic (AC7 overexpression), whole brain and pituitary; WT/AC7 Heterozygote, whole brain and pituitary; WT/Fyn KO; C57BL/FVB F1 <u>Selected rat lines:</u> AA/ANA; IHAS/ILAS (2 replicate lines); IP/INP (multiple brain regions); P/NP; HAD/LAD (2 replicate lines); sP/sNP; UChA/UChB</p> <ul style="list-style-type: none"> • 4–6 animals per strain or line (one animal/array) • Age 10–14 weeks; separate male and female databases • Data in precompiled databases have been normalized and quality controlled; data can be renormalized to include all or selected strains • Data can be combined with user-uploaded data • MIAME information for all arrays • Affymetrix M430v2 or exon arrays (mice); Codelink arrays (rats) 	<p><u>Precompiled Public Databases</u> BXD RI mice – whole brain (32 strains obtained from Phenogen); cerebellum (30 strains); hippocampus (69 strains); neocortex (53 strains); nucleus accumbens (36 strains); prefrontal cortex (29 strains); striatum (31/56 strains). CXB RI mice: hippocampus (15 strains). LXS RI mice: hippocampus (77 strains); prefrontal cortex (42 strains). HXB/BXH RI rats: hippocampus (32 strains).</p> <ul style="list-style-type: none"> • Data have been processed (normalized and summarized as strain means) • Most databases consist of 2 arrays/strain, one from pooled samples of females, one from pooled samples of males; data from individual arrays cannot be removed; age range 41–196 days or not provided. • Affymetrix M430v2 or exon arrays; Illumina 6.1 arrays
Phenotype Data	
<ul style="list-style-type: none"> • Data (from literature, or user-supplied) can be uploaded 	<ul style="list-style-type: none"> • Data can be uploaded, or precompiled databases of publicly accessible data can be used
QTL Calculations	
<u>Behavioral (phenotypic) QTL</u>	<u>Behavioral (phenotypic) QTL</u>
<ul style="list-style-type: none"> • Marker regression analysis, genome-wide P values by permutation • 3 methods to calculate confidence intervals • Calculations for all RI panels on website 	<ul style="list-style-type: none"> • Marker regression analysis, genome-wide P values by permutation • Interval mapping • Calculations for all RI panels on website.
<u>Expression QTL</u>	<u>Expression QTL</u>
<ul style="list-style-type: none"> • Database created with advanced methodology (see User Manual) 	<ul style="list-style-type: none"> • Processed based on user input, flexibility on analysis method.

* Gene expression data for other tissues and species are also available on each website in public or private databases. Private databases on PhenoGen can be accessed with permission of the responsible investigator. See Supplementary Discussion for more detail.